



# THE MECHANISM OF ACTION OF INSULIN

A SYMPOSIUM

organized by

THE BRITISH INSULIN MANUFACTURERS

*Allen & Hanbury Ltd  
British Drug Houses Ltd*

*Boots Pure Drug Co Ltd  
Bristol Wilcome & Co Ltd*

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\* Session Chairman  
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## INTRODUCTION

F G YOUNG

It is at once a salutary fact and a challenge to research that nearly 40 years after the discovery of insulin much disagreement still exists about the mechanism of action of this important hormone. The annual volume of publications on this subject is enormous and is still rapidly growing nevertheless ideas about the way in which insulin acts appear to steer an unsteady course between the Charybdis of sugar transport and the Scylla of direct action on identifiable enzyme systems with occasional sidelong glances at other possibilities. Perhaps in the end Charybdis will engulf Scylla and we shall understand the action of insulin in terms of a promotion of transport of glucose into a cell by virtue of an influence of insulin on certain enzymes or co-enzymes which are themselves concerned with the transport of sugar across permeability barriers of the cell. Already a few rays of light shine in this hopeful direction and the account of the contributions and discussions recorded in this volume shows that there need be no deep dissatisfaction with the progress made in recent years.

Although meetings for the discussion of current research and the subsequent publication of a record of the contributions and discussion are becoming not uncommon these days the meeting which this volume is the published record differed from many others in two respects. The subject of discussion was in theory a narrow one although in practice the discussions were wide while the taking part included both chemists and clinicians with a common interest. Insulin was the focus of all providing the cohesive force which held the meeting together in vigorous discussion for successive days.

The absence of Professor Loubatières who was kept away last moment by family illness was most disappointing but present were deeply indebted to Rachmael Levine for filling in the last moment and giving a masterly survey of the Professor Loubatières and others on the action of anti-sulphonamides.

Although a full record of the discussions was made the of the meeting agreed at the beginning that anybody was excise from the record remarks which on due reflection ferred not to be recorded in print. With this in mi



urged to be informal and frank in their discussions and not to hesitate to ask simple questions as well as those that might display the erudition of the questioner. The simple questions can sometimes be the most penetrating. How far this exhortation was effective can be seen from the following pages but it is refreshing to note that very little of the recorded discussions was in fact excused by the contributors.

The expenses of the meeting including much of the travel costs of the members were provided by the British Insulin Manufacturers. This organization was formed as the result of an agreement between the four manufacturers of insulin in Great Britain (Allen & Hanburys Ltd, Boots Pure Drug Co Ltd, British Drug Houses Ltd and Burroughs Wellcome & Co Ltd) to provide for exchange of information and the fostering of research on insulin. The conference was the first held under the auspices of the British Insulin Manufacturers of which an account is published. The editing of the record was undertaken jointly by Mr W. A. Broom of Boots Pure Drug Co Ltd and Dr F. W. Wolff of Burroughs Wellcome Ltd. Dr Wolff also undertook most successfully the detailed administrative duties involved in the organization of the conference. On behalf of the British Insulin Manufacturers the Chairman of their Technical Committee, Dr G. I. Hobday of Boots Pure Drug Co Ltd, opened the meeting which was held in the Wellcome Building, London on September 9th and 10th 1958.

All those present hoped that the first meeting of this sort will not prove also to be the last and the published record may reveal some of the reasons for this hope to those concerned in some way or another with the substance that has saved more lives and made an almost normal life possible for more people than any other hormone. As was said at the meeting it is indeed fortunate that ability to use insulin therapeutically in no way depends on a detailed knowledge of its mechanism of action. But nobody can properly doubt that growth in the understanding of insulin and its ways will inevitably bring with it a wider and more effective therapeutic application. The more the laboratory worker and the clinician understand each other's tasks the more quickly the results of research can become fruitful in the treatment of disease. This book is published as a contribution to this end.

PART I  
CHEMICAL NATURE OF INSULIN  
Chairman PROFESSOR F G YOUNG



# THE ACTIVITY OF MODIFIED FORMS OF INSULIN

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The purpose of testing the activity of chemical structures related to insulin is to discover the essential site of activity in the molecule as this may help towards revealing the mechanism of its action. Another aspect of investigations of this nature which will not be dealt with here is the important one of discovering modifications which are more valuable and convenient in the treatment of diabetes.

Although retrospective in time Sanger's formula of insulin<sup>37</sup> will help us to describe briefly previous results in the main field of re-

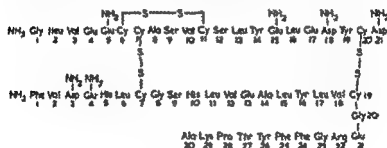


FIG. 1. The structure of insulin.

lating structure to activity (Fig. 1). Various reviews are on this subject. Among them are those of Anfinsen and Redf. Li<sup>38</sup>, Porter<sup>39</sup> and Sanger<sup>39</sup>. A prepared table (see Table I) of the distinguished work of the past 35 years in the modification of insulin would lead to the conclusions that the essential groups or linkages were the free carboxyl group, the hydroxyl groups and the disulphide bonds, and that the essential bonds were the amino groups, the guanidino, the amide groups and the aliphatic hydroxyl groups.

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When soon after its discovery insulin was shown to be a protein modification of the molecule by proteolytic enzymes was extensively investigated. The use of proteolytic enzymes was important because of their specific nature and the gentle conditions under which they acted.

The discoverers of insulin Banting and Best<sup>4</sup> had shown that trypsin destroyed insulin. In fact the diminution or absence of proteolytic enzymes from the pancreas was one of the factors which they made use of in their isolation of insulin. Other workers confirmed this and the inactivating nature of pepsin and papain.<sup>10 41 4 47</sup> Epstein and co-workers did not believe the action of trypsin was proteolytic and claimed to have reactivated insulin which had been subjected to trypsin digestion for 42 hours.<sup>11</sup> Their work could not be confirmed, however,<sup>41</sup> probably because their criterion of insulin action — convulsions in a rabbit — was too diffuse.

The tryptic and peptic hydrolyses of insulin were further studied in detail by Charles *et al*.<sup>9 15</sup> at the Connaught Laboratories in Toronto and by Freudenberg and his co-workers in Germany.<sup>16</sup> Attempts were made to correlate the destruction of physiological activity with the loss of protein. The former was found to exceed the latter greatly.

More recently Butler *et al*.<sup>5 6 7</sup> showed that crystalline chymotrypsin, trypsin and pepsin destroyed the activity of insulin. Van Abele and Campbell<sup>1</sup> found that under mild conditions trypsin did not destroy the activity of insulin and Harris and Li<sup>2a</sup> confirmed this showing that only alanine was released.

Lens<sup>8</sup> showed the release of alanine from insulin by carboxypeptidase and with this he found that most of the activity had disappeared. Harris and Li<sup>2a</sup> probably using a more purified sample of enzyme found that activity was retained in the molecule after alanine was removed but that further action resulted in the removal of asparagine and a diminution in the biological activity of insulin.

It was at this point that the formula of insulin was finally and completely elucidated by Sanger and his team at Cambridge.<sup>37</sup> This opened the way to a more exact assessment of results in this type of investigation. Since then Smith *et al*.<sup>43</sup> have shown that leucine aminopeptidase removes a considerable number of amino acids from the N-terminal end of insulin without complete impairment of its activity.

Our own investigations were directed towards the preparation of

insulin peptides of known structure which could be tested by the rabbit blood sugar method and by the glucose uptake of isolated rat hemidiaphragms. Fisher's *t* test was always applied to the latter.

The action of carboxypeptidase on insulin was first investigated. The enzyme was first incubated with DFP (Di-isopropylfluorophosphoridate) which inactivates any contaminating trypsin and chymotrypsin present. Insulin was then added and the enzyme-substrate mixture incubated at pH 7.8. Conditions were obtained in which most of the C-terminal alanine was removed but very little of the asparagine. When this was done the activity was unaffected (Fig. 2). When however a greater quantity of asparagine was removed there was diminution in biological activity (Fig. 3). The work of Harris and Li was thus confirmed.

Sanger and Thompson<sup>40</sup> had shown that the action of carboxypeptidase was more effective with acetyl-insulin as substrate than with insulin. Acetyl-insulin (when only the amino groups are acetylated) is active (see Table I). The peptide resulting from the removal of alanine and asparagine by the action of carboxypeptidase showed considerable loss of activity (Fig. 4).

The action of trypsin on insulin is at two sites on the phenylalanyl chain (Fig. 1). These are beyond the arginyl residue<sup>2,23</sup> and beyond the lysyl residue<sup>29,30</sup>. Thus the three products of hydrolysis are a large peptide (DHA insulin we have called it) a heptapeptide Gly Phe Phe Tyr Thr Pro Lys and the terminal alanine molecule. Different conditions were used to increase the extent of the hydrolysis. The most successful was when the substrate used was insulin which had been treated with versene to remove most of its content of zinc. It will be recalled that Smith *et al.*<sup>42</sup> first used this low content zinc insulin in their leucine-aminopeptidase experiments. After incubation for about 24 hours the tryptic hydrolysate was then brought to pH 5. The supernatant of the tryptic hydrolysate contained the heptapeptide and alanine (Fig. 5) and these were separated by ionophoresis and identified by paper chromatography after acid hydrolysis. The precipitate contained both DHA insulin and unchanged insulin (Fig. 6). Separation of both was achieved by an Andersen type column<sup>2</sup>. The integrity and composition of DHA insulin was determined by various methods including an analysis by the Moore-Stem method carried out by Mr M. Rees and Miss B. Worboys of this department and by the DNP N-terminal analysis method<sup>28</sup>.

TABLE I

THE ACTIVITY OF SOME CHEMICALLY MODIFIED FORMS OF INSULIN

Authors	Procedure	Groups affected	Activity
Freudenberg <i>et al</i> (1931) <sup>18, 19</sup>	Acid hydrolysis	Peptide bonds	Inactive
Phillips (1952) <sup>24</sup>	Acid hydrolysis (Conc. HCl)	Peptide bonds	Inactive
Mills (1954) <sup>21</sup>	Acid hydrolysis (Conc. HCl)	Peptide bonds	Inactive
du Vigneaud <i>et al</i> (1931) <sup>27</sup>	Reduction	S-S-bridges	Inactive
Freudenberg <i>et al</i> (1935) <sup>19</sup>	Reduction	S-S-bridges	Inactive
Stern and White (1937) <sup>43</sup>	Reduction	S-S-bridges	Inactive
Freudenberg <i>et al</i> (1932) <sup>8</sup>	Oxidation	S-S-bridges	Inactive
Freudenberg <i>et al</i> (1935) <sup>19</sup>	Oxidation	S-S bridges	Inactive
Fraenkel-Conrat J and H (1950) <sup>4</sup>	Reduction	S-S bridges	Inactive
Harington and Neuberger (1936) <sup>1</sup>	Iodination	Phenolic hydroxyl groups	Inactive
Stern and White (1938) <sup>48</sup>	Acetylation	Phenolic hydroxyl groups	Inactive
Fraenkel-Conrat J and H (1950) <sup>4</sup>	Iodine pH 7.6 - hr	Phenolic hydroxyl groups	Inactive
Fraenkel-Conrat J and H (1950) <sup>14</sup>	Phenylisocyanate in chlorophenylisocyanate	Phenolic and imidazole groups - extensive	Inactive
Fraenkel-Conrat J and H (1950) <sup>14</sup>	Diazobenzenesulphonic acid	A few phenolic and imidazole groups	Partially active
Jensen <i>et al</i> (1928) <sup>24</sup>	Acetylation	Amino and others	Inactive
Freudenberg <i>et al</i> (1928, 1932) <sup>18, 19</sup>	Acetylation	Amino hydroxyl amino	Partial reversible inactivation
Hopkins and Wormald (1934) <sup>20</sup>	Phenylisocyanate p-bromophenyl isocyanate	Free amino groups	Inactive
Jensen and Evans (1934) <sup>25</sup>	Phenylisocyanate	Amino and hydroxyl groups	Inactive
Stern and White (1938) <sup>48</sup>	Acetylation	Free amino groups	Active
Fraenkel-Conrat J and H (1950) <sup>14</sup>	Acetic anhydride	Amino groups	Active
Fraenkel-Conrat J and H (1950) <sup>14</sup>	Ketene	Amino groups	Active
Fraenkel-Conrat J and H (1950) <sup>14</sup>	Mutaric pH 4.1 30 min	Amino groups	Active
Li (1956) <sup>20</sup>	2,4-dinitrobenzene sulphonate	Amino groups of Lysine	Active
Reitz <i>et al</i> (1946)	Sulphonation (Conc. H <sub>2</sub> SO <sub>4</sub> at -18°)	Aliphatic hydroxyl groups of serine and threonine	Active
Fraenkel-Conrat J and H (1950) <sup>4</sup>	Phosphorylation	Aliphatic hydroxyl groups	Partially active
Freudenberg <i>et al</i> (1932) <sup>19</sup>	Formaldehyde	Reduction	Inactive
Scott (1955)	Formaldehyde	Reduction	Inactive
Fraenkel-Conrat and Olcott (1948) <sup>12</sup>	Formaldehyde at pH 11.12	Amido Guanidino } groups	Active
Carr <i>et al</i> (1929) <sup>6</sup>	Esterification	Free carboxyl groups	Inactive
Freudenberg <i>et al</i> (1931) <sup>18</sup>	Esterification	Free carboxyl groups	Inactive
Glendennin <i>et al</i> (1947)	Esterification	Free carboxyl groups	Inactive
Mommmaerts and Neurath (1950) <sup>22</sup>	Esterification	Free carboxyl groups	Inactive
K plan <i>et al</i> (1944, 1950) <sup>26</sup>	Ultra violet radiation	Denaturation	Inactive
Phillips (1952) <sup>24</sup>	Ether trichloroacetic acid 5M urea, diisopropyl fluorophosphonate	Physico chemical combination	Active

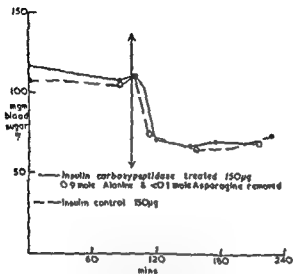


FIG. 2. The peptide resulting from the action of carboxypeptidase on insulin (I) — rabbit blood-sugar method.  
Time of intravenous injection

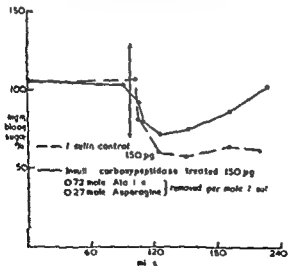


FIG. 3. The peptide resulting from the further action of carboxypeptidase on insulin (II) — rabbit blood-sugar method.  
Time of intravenous injection.



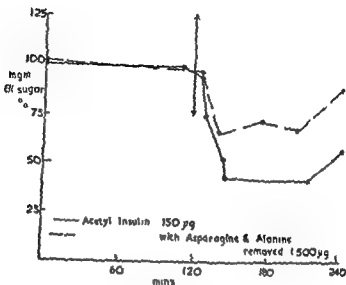


FIG 4 The peptide resulting from the action of carboxypeptidase on acetyl insulin - rabbit blood-sugar method  
Time of intravenous injection

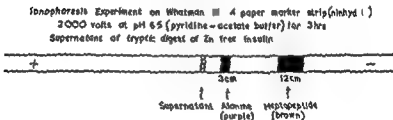


FIG 5 The supernatant fluid from the tryptic digest of insulin at pH 6.2

The assay of the first isolated batch of DHA insulin showed little activity<sup>33</sup>. But gentler methods of extraction of the column fluid followed by confirmatory analysis<sup>33a</sup> gave samples of DHA insulin which showed about 15 per cent activity (see for example Fig 7). The heptapeptide showed no activity (Fig 8) neither did a mixture of DHA insulin heptapeptide and L-alanine give more than the level of DHA insulin alone (Figs 9 and 10). Complete esterification was

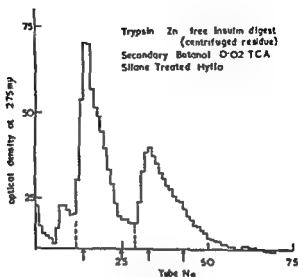


FIG. 6 Separation of DHA insulin (first peak) from unchanged insulin by reverse phase column chromatography

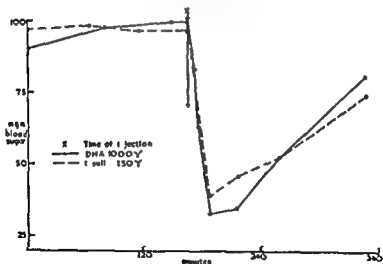


FIG. 7 The effect of DHA insulin—rabbit blood-sugar method.  
Time of intravenous injection.

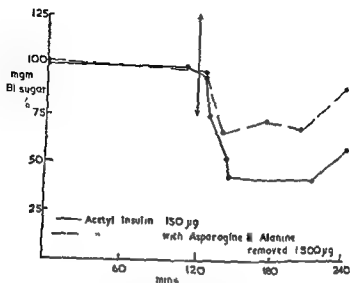


FIG 4 The peptide residues from the action of carboxypeptidase on acetyl insulin - rabbit blood sugar method  
Time of intravenous injection.

Isophoresis Experiment on Whatman N 4 paper marker strip (ninhydrin)  
2000 volts at pH 5.5 (pyridine-acetate buffer) for 3 hrs  
Supernatant of tryptic digest of Zn free insulin

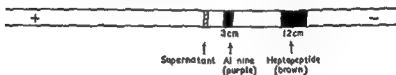


FIG 5 The supernatant fluid from the tryptic digest of insulin at pH 5.2

The assay of the first isolated batch of DHA insulin showed little activity<sup>33</sup> But gentler methods of extraction of the column fluid followed by confirmatory analysis<sup>33a</sup> gave samples of DHA insulin which showed about 15 per cent activity (see for example Fig 7). The heptapeptide showed no activity (Fig 8) neither did a mixture of DHA insulin heptapeptide and l-alanine give more than the level of DHA insulin alone (Figs 9 and 10). Complete esterification was

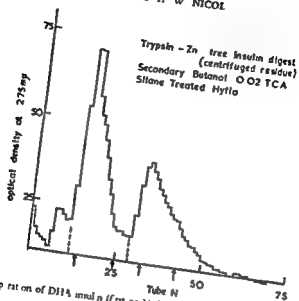


FIG 6 Separation of DHA insulin (first peak) from unchanged insulin by reverse phase column chromatography

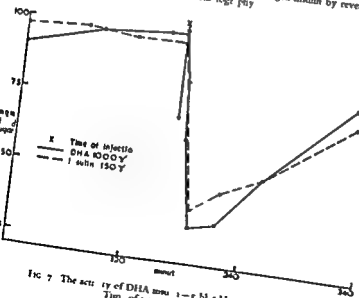


FIG 7 The activity of DHA insulin - a blood-sugar method.  
Time of intravenous injection.

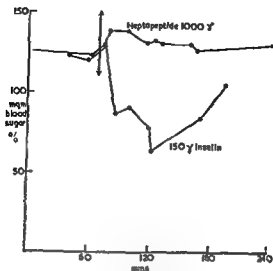


FIG. 8 The activity of the heptapeptide Gly Phe Phe Tyr Thr Pro Lys — rabbit blood sugar method  
Time of intravenous injection.

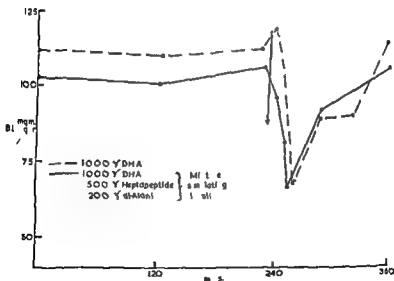


FIG. 9 The activity of a mixture of DHA insulin, the heptapeptide and dl alanine compared with that of DHA insulin — rabbit blood sugar method.  
Time of intravenous injection.

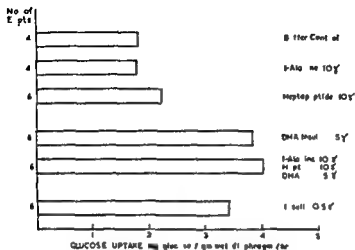


FIG. 10 The activities of the substances resulting from tryptic digestion of insulin, separately and together. Glucose uptake of r 1 diaphr gm method.

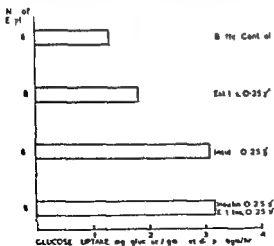


FIG. 11 The activity of extracted insulin alone and with insulin. Glucose uptake of r 1 diaphr gm method.

carried out by methanolic hydrochloric acid and it was confirmed that activity was lost (Fig 11)

A sample of acetyl insulin donated by Dr L F Smith in which the amino groups but not the hydroxyl groups of tyrosine had been acetylated showed activity Esterified and acetylated insulin have of course been investigated before by other workers (see Table I) and our work has simply confirmed their results using the new assay technique of the glucose uptake of the isolated rat diaphragm

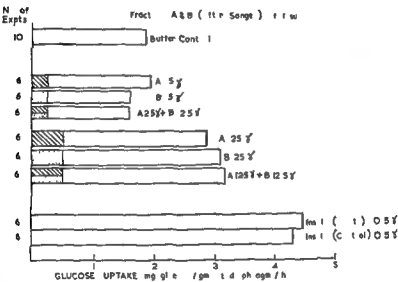


FIG 12 The activity of Fractions A and B (Sanger) on insulin separately and together  
Glucose uptake of rat diaphragm method

The oxidized glycol and phenylalanyl chains (Fractions A and B) were prepared and purified by electrophoresis A single experiment showed (Fig 12) an increase in the glucose uptake of the isolated diaphragm but at a level a hundred times less than insulin Together Fractions A and B gave only an additive and not a synergistic effect (Fig 12)

Finally these experiments chiefly those connected with the proteolytic hydrolysis of insulin show the importance of asparagine in the glycol chain and of the heptapeptide in the phenylalanyl chain

the maintenance of the full activity of insulin. This may reflect sensitive and essential nature of the region around the second interchain disulphide linkage of insulin

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## DISCUSSION

YOUNG I think it has been the hope of many people for many years that the existence of a prosthetic group of insulin might ultimately be revealed but since the work of Sanger and his colleagues has elucidated the complete chemical structure of insulin the hopes of finding a prosthetic group have tended to fade. Dr Nicol's work done as it is with chemically purified fractions of insulin has in fact shown that the integrity of a number of parts of the molecule is essential for biological activity.

This paper is now open for discussion.

KRAHL Does Dr Nicol have any information about effect of reduction of the disulphide bonds in the A or B chains on activity of insulin?

NICOL I think a former colleague of ours at Cambridge Dr Ryle with Dr Sanger did some work on the reduction of these disulphide linkages but it was not quite clear from their work or the work of others in the United States and at Oxford which of the disulphide linkages definitely is the most important. It seems that as soon as one of them goes activity drops suddenly it not entirely. Perhaps someone else here might have some contribution on that topic.

PETROW Willie has recently drawn attention to the fact that in these large polypeptide structures the actual units often are not important and that for example in the vasopressin type you can interchange amino acids. It seems to me that with this work on insulin we are approaching the stage when it will be possible to synthesize an appropriate ring structure the actual amino acids in the ring will probably not be very important apart from certain amino acids of the type Dr Nicol has drawn attention to and then you would have to add the short chains to the sides. I know that is looking ahead a good bit but I do think from what has been said this morning that we are actually getting near this point.

RENOLD I should like to ask Dr Nicol what his prediction would be concerning the biological activity of insulin which has been iodinated. I believe that this would be expected to alter the characteristics of the tyrosine groups?

NICOL Yes it would alter the tyrosine molecule. With work on acetylation in covering of the hydroxyl groups the essential nature of the hydroxyl groups in that molecule has been shown. Any alteration of activity which there may be in the iodinated insulin I should imagine would have to do with the essential nature of the aromatic amino-acid tyrosine.

SCIAMBYE. Is the removal of zinc from the insulin molecule by versene conditional for the effect you obtained by the trypsin digestion or liberation?

Secondly how completely was the zinc removed from the insulin molecule before you obtained the effect of trypsin?

NICOL. Without the removal of zinc trypsin does act on insulin. It hydrolyses it to the extent of about 15 per cent of the total quantity of polypeptides you would expect removed. However you do not obtain enough of the DHA insulin to make separation easy. When the zinc content is reduced and it was reduced from about 0.5 per cent content to 0.25 per cent the extent of hydrolysis was more than doubled and enough DHA insulin was obtained to make the separation easier. It may be that the insulin molecule as such is composed of two of the units shown and zinc may combine both units through attachment perhaps to the histidine molecule as has been suggested perhaps the removal of the zinc renders the molecule more easily to the enzymic hydrolysis of trypsin but that is just a conjecture.

Of course Dr Emil Smith of Salt Lake City Utah first showed that the action of leucine amino peptidase was increased on relatively zinc free insulin. Our removal of zinc by versene was carried out by a method which was first published in 1955 by Sluytermann (*Biochim biophys acta* 17 169).

BEHRENS. You have reported that the separated A and B chains have low order of activity when tested on the rat diaphragm insulin assay. Have you had an opportunity to study any other long chain polypeptides to see whether they share this property or is it unique for these fragments of insulin? Is there any possibility that such insulin fragments may be responsible for some of the insulin like effects on the rat diaphragm that are observed in plasma?

NICOL. We have not tested any long chain polypeptides of this nature or some different substances other than insulin or insulin related molecules. Of course give an increased glucose uptake. The interest these two have for us is that one has to use such an unphysiologically high quantity of 5 micrograms to obtain an effect which you could get from insulin in our hands with about 0.1-0.5 micrograms of insulin.

KORNER. May I take issue with Dr Petrow on his suggestion that the amino-acid sequence of a protein is not a unique sequence for that protein and that apart from a few important sites, the amino-acid composition is random?

Sanger has analysed insulin molecules from five different species and has shown that they differ from one another only in minor changes in amino-acid sequence at one point in one of the peptide chains. Ingram has demonstrated that the only difference in the haemoglobin molecules of

blood from normal people and from sickle-cell patients lies in a change of only one amino acid in over three hundred present in the molecule. Yet this produces enormous changes in the chemical and physiological behaviour of the whole molecule. These are but two examples from the large body of evidence accumulating and which shows that the amino-acid sequence of a protein is uniquely determined and is genetically controlled.

PETROW Dr Korner broadly supports my view obviously because my point is that you have got certain sequences of amino acid which are very important attached to a ring which may itself have few prosthetic groups or important amino acids. I believe what is required is a large ring of the right size and that there will be a fair amount of permutation possible with the unimportant amino acids in that ring.

PART II

INSULIN AND TRANSPORT SYSTEMS

*Chairman* PROFESSOR I G YOUNG



# THE MEMBRANE TRANSPORT OF SEVERAL MONOSACCHARIDES IN HEART MUSCLE AND THE REGULATION OF THIS PROCESS BY INSULIN ANTERIOR PITUITARY AND ADRENAL CORTICAL HORMONES\*

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In this paper we would like to report some observations on the passage of glucose and several other sugars through the cell membrane in muscle. Two questions have been investigated: first, do sugars enter the cells by diffusion or by a specific transport system which requires interaction between the sugar and the membrane? Second, to what extent can the hormonal control of glucose utilization be explained by regulation of the entry process? For such studies an *in vitro* muscle preparation is most desirable since close control of sugar and hormone levels is possible. With these considerations in mind we have employed the isolated rat heart perfused through the coronary circulation by a technique analogous to that described by Bleehen and Fisher.<sup>1</sup>

The particular advantages of the perfused heart compared to other *in vitro* preparations are: first, sugar and hormones reach the cells rapidly by the intrinsic circulatory system of the muscle. Second, all substances enter the cell through the membrane itself since there are no cut edges. Third, the muscle remains in a good physiological condition as can be directly observed by the rate and force of the heart beat.

## THE KINETICS OF THE TRANSPORT PROCESS

The penetration of a non-metabolized sugar through the membrane has been estimated by the accumulation of the sugar within the cell after a suitable period of perfusion. The accumulation has been measured by determining the total sugar content of the tissue and subtracting the fraction which is extracellular. Labelled sor-

\*This work was supported by grants from the National Science Foundation, the U.S. Public Health Service and the American Heart Association.

Part I of this work has been presented earlier in preliminary form.

bitol an alcohol closely related in physical properties to glucose itself was used to measure the extracellular volume. As shown in Fig. 1 sorbitol distributes in a volume corresponding to 30 per cent of the tissue by weight. This value is essentially constant after about 5 minutes up to periods as long as 2 hours as determined in recent

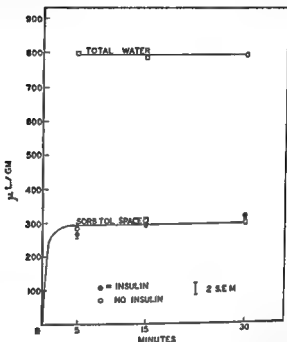


FIG. 1. The distribution of sorbitol and the water content in the isolated perfused rat heart. The curve has been drawn for the no insulin points  $\pm$  S.E.M. in the abbreviation for twice the standard error of the mean.

The heart was perfused with sorbitol  $^{14}\text{C}$  and carrier to give a sorbitol concentration of 50 mg per cent in the perfusate. The heart was extracted as described earlier<sup>11</sup> and the sorbitol content determined by estimation of radioactivity using a liquid scintillation counter. The sorbitol or extracellular volume was calculated as follows:

$$\text{ECV } (\mu\text{L/gm}) = \frac{\text{Mean Conc Sorb in tissue } \text{H}_2\text{O}}{\text{Conc Sorb in perfusate}} \times \text{water content of tissue } (\mu\text{L/gm})$$

The amount of extracellular sugar in any experiment was calculated by multiplying the ECV by the perfusate concentration of the sugar. Intracellular sugar was determined as the total sugar minus the extracellular sugar.

In these and all subsequent experiments to be described the perfusion medium was Krebs-Henseleit carbonate buffer<sup>8</sup> gassed continuously with 95 per cent  $\text{O}_2$  and 5 per cent  $\text{CO}_2$ . The temperature was maintained at 37.

In these and subsequent experiments in which hearts from normal rats were used the animals were fasted about 18 hours.

experiments. The distribution of sorbitol was measured in every experiment discussed below and was unchanged by hormonal or other experimental variables except for an increase of a few per cent when very high concentrations of sugar were added to the perfusion fluid.

The rapid rate at which substances such as sugars exchange in the extracellular space of the heart preparation could be demonstrated by measuring the washout of labelled sorbitol. After the extracellular water had first come into equilibrium with sorbitol during a period of 15 minutes (see Fig. 1) the medium was then changed

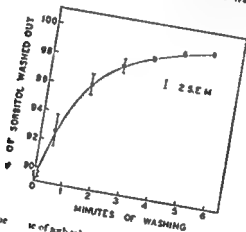


FIG. 2. The time course of sorbitol wash-out in the isolated perfused heart. See text for explanation.

to sorbitol free buffer which was passed a single time through the heart and was collected during successive minute intervals for sorbitol analysis. After 1 minute of this washing 93 per cent of the alcohol had been removed and after 5 minutes the washout was 99 per cent complete (see Fig. 2).

The experiments of Fig. 3 suggest that the entry of sugar into the cell does not occur by simple diffusion. The intracellular accumulation of 3-O-methyl glucose, a non-metabolizable sugar, was determined after a fixed period of perfusion at various perfusate concentrations. The solid lines show that a non-linear relationship of intracellular concentration to perfusate concentration was found in



both the control and insulin series. If the penetration of the membrane occurred by simple diffusion it would be expected that the intracellular concentration would be directly proportional to the perfusate concentration. It can be seen however, that the ratio of the intracellular to perfusate concentration decreased as the perfusate concentration increased the values falling from about 0.6 to 0.3 in the control and from 0.8 to 0.5 in the insulin series. These kinetics suggest progressive saturation of combining sites for the sugar; this would indicate that transport involves an interaction of the sugar

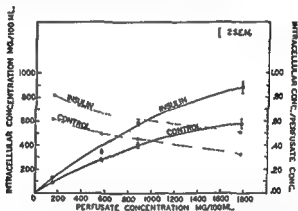


FIG. 3 The relationship of intracellular accumulation of 3-O-methyl glucose to the concentration of the sugar in the medium

Hearts from normal rats were perfused in all cases for 30 minutes. No glucose or other metabolizable substrate was present in the medium. No visible impairment of function of the muscle has been noted under these conditions up to periods of 1 hour. 3-O-methyl glucose was determined by the Nelson<sup>8</sup> procedure after extraction and deionization as described earlier.<sup>21</sup>

with the membrane. Similar observations have been made in the case of the erythrocyte and a discussion of their significance is to be found in a recent review by Bowyer.<sup>2</sup>

More definitive evidence for interaction between the sugar and a membrane component was obtained by determining the presence of competition for transport between sugars. No competition would be anticipated in a simple diffusion system. In the control experiment at the top of Table I the intracellular accumulation of a non-metabolizable pentose L-arabiose is shown after perfusion for 15 minutes at a concentration of 500 mg per cent. The addition

an equimolar concentration of 3-O-methyl glucose to the medium inhibited arabinose penetration by about 86 per cent. Glucose was an equally effective inhibitor. L-arabinose transport was accelerated by insulin and the accelerated system could also be inhibited by glucose although the extent of inhibition apparently was not as great. In the lower panel it can be seen that the penetration of glucose was also strongly inhibited by glucose.

TABLE I

THE INHIBITION OF L-ARABINOSE AND D-XYLOSE TRANSPORT BY 3-O-METHYL GLUCOSE AND GLUCOSE

L-arabinose was determined as described earlier.<sup>11</sup> The lack of L-arabinose and 3-O-methyl glucose utilization was determined in separate experiments in which measurable loss of sugar was observed when about 4 ml. of medium were re-irradiated through the heart for 30 to 60 minutes.

Sugar added and molar ratio L-arabinose	Insulin $\mu$ g/ml	Intracellular concentration of Pentose $\pm$ S.E. %
Perfusion for 25 minutes with medium containing 500 mg per cent L-arabinose with addition of other sugars as indicated		
None	0	157.1 $\pm$ 10.0 (16)
3-O-methyl glucose 1:1	0	21.8* $\pm$ 9.0 (7)
Glucose 1:1	0	38.0 $\pm$ 7.5 (8)
None	+	207.2 $\pm$ 6.6 (14)
Glucose 1:1	+	157.1* $\pm$ 19.1 (7)
Perfusion for 10 minutes with medium containing 400 mg per cent D-xylose with addition of other sugars as indicated		
None	0	123.0 $\pm$ 12.0 (6)
Glucose 1:25	0	40.6 $\pm$ 11.0 (6)

\*  $P < .005$  vs. respective control

Table II shows conversely that glucose transport can be inhibited by L-arabinose and 3-O-methyl glucose. In the absence of insulin the transport of glucose is indicated by the utilization of the sugar from the medium. Despite appreciable transport into the cell it can be seen that the free glucose distribution remained less than the sorbitol or extracellular space of 300  $\mu$ l/gm. As discussed in detail in earlier papers<sup>11,12,18</sup> this indicates that the glucokinase system

keeps pace with glucose entry under these conditions. With the addition of insulin however transport was greatly increased as shown by the rise in utilization and now the free glucose space considerably exceeded the sorbitol space. This can be most readily interpreted as due to an acceleration of transport to the point where the entry of glucose is so rapid that it exceeds the capacity of the glucokinase system. The next lines of the table show that L-arabinose was a poor inhibitor of glucose transport: a 4:1 molar ratio of pentose to glucose did not appreciably affect utilization and even a

TABLE II

## THE INHIBITION OF GLUCOSE TRANSPORT BY L-ARABINOSE AND 3-O-METHYL GLUCOSE

In all experiments glucose was perfused at a concentration of 150 mg per cent for 15 minutes. Glucose was measured as previously described<sup>13</sup> using the hexokinase-zwischenferment method to ensure specificity.

Sugar added and molar ratio to glucose	Insulin 0.3 $\mu$ g/ml	Utilization mg/g/l/r $\pm$ SEM	Glucose space $\mu$ l/g $\pm$ SEM
None	0	4.5 $\pm$ 0.5 (10)	227 $\pm$ 9
None	+	9.6* $\pm$ 0.5 (15)	458* $\pm$ 14
L-arabinose 4:1	+	9.7 $\pm$ 1.0 (10)	470 $\pm$ 14
L-arabinose 12:1	+	7.4* $\pm$ 0.6 (11)	385* $\pm$ 12
3-O-methylglucose 4:1	+	5.6* $\pm$ 1.3 (5)	275* $\pm$ 17
3-O-methylglucose 8:1	+	4.6* $\pm$ 1.0 (5)	272* $\pm$ 5

\* P < 0.05 vs appropriate control

12:1 ratio was not strongly inhibitory. 3-O-methyl glucose on the other hand inhibited strongly at 4 and 8:1 molar ratios. In all instances where utilization was reduced the glucose space was also reduced. This is evidence that competition takes place at the transport step since inhibition of metabolism within the cell should cause an increase in space due to a rise in intracellular free glucose.

In earlier studies with the erythrocyte it was possible to show that competition between sugars can lead under certain conditions to transport of one of the sugars against a concentration gradient.<sup>13</sup> This active transport could also be shown in the case of the heart

muscle (Fig. 4). When the tissue was perfused with 150 mg per cent of 3-O-methyl glucose the intracellular concentration rose to about 86 mg per cent in the first 5 minutes and then increased slowly to about 93 mg per cent during the next 25 minutes. However, when glucose was added to the medium at the 5-minute point the intracellular 3-O-methyl glucose decreased, the extent of fall being dependent on the amount of glucose added.

With the addition of a tenfold concentration of glucose the intracellular 3-O-methyl glucose was reduced to 34 mg per cent, that is

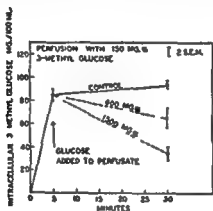


FIG. 4. The active transport of 3-O-methyl glucose out of the cell due to competition by glucose for the transport system.

The concentration of 3-O-methyl glucose in the perfusate was maintained at 150 mg per cent throughout the experiment. The concentration of glucose added is indicated by the values above the broken lines.

to 40 per cent of the control value. Since 3-O-methyl glucose could not be metabolized, it must have been transported out of the cell, although this movement was against the concentration gradient. This can be most easily explained by depression of 3-O-methyl glucose influx due to the competitive activity of glucose for the transport system at the external surface of the membrane. Inside the cell, however, the glucose was rapidly metabolized and was therefore less effective as a competitor for the efflux process. As a result, efflux transiently exceeded influx and a net movement took place of 3-O-methyl glucose out of the cell. A competition effect of this

kind is readily explained on the basis of a carrier sugar transport and is difficult to account for by other (see discussion by Bowyer<sup>2</sup>)

On the basis of these competitive studies the order of the various sugars for the membrane-combining site is glucose > 3-O-methyl glucose > xylose and L-demonstration in these competition studies that non-metabolizable sugars employ the same transport also provides valuable support for the assumption of authors that transport data obtained with these sugars can be used to draw conclusions regarding the glucose itself. In regard to the effect of insulin it may be that although the hormone accelerates transport it does not change the system to one of simple diffusion since the kinetics are of the saturation type and competition between sugars is still observed. The absence of an added diffusion element in the insulinized heart is also indicated by the failure of sorbitol to enter the cell (see discussion) although this substance is very similar to glucose with respect to size and lipid solubility.

The present observations are in harmony with the earlier work of Fisher *et al*<sup>3,4</sup> showing that galactose penetration in the heart does not conform to the kinetics of simple diffusion and that galactose and glucose are competitive for the transport process.

#### THE EFFECT OF VARIOUS HORMONAL FACTORS ON MEMBRANE TRANSPORT

While the acceleratory effect of insulin on sugar transport is well established from work in many laboratories, very little is known regarding the possible inhibitory effects of other hormones on the transport process, although such effects would be expected from earlier studies of glucose uptake. Preliminary results on the presence of transport inhibitors in the muscle from alloxan-diabetic rats and also from normal animals is presented in Table III. Since glucose, which is rapidly metabolized, was used as the test sugar, it was necessary to evaluate transport rates indirectly by measurement of the utilization and distribution of the sugar as discussed below. Data obtained with hearts from normal animals with and without insulin are shown for reference in the first two lines. The glucose utilization by the heart from alloxan-diabetic rats was severely depressed, but the glucose distribution, however, remained approximately equal to the sorbitol space. This indicated that the inhibition of utilization

as at the transport step since if hexokinase had been inhibited glucose should have accumulated within the cell. These tentative conclusions could be supported and extended using a more direct and sensitive experimental method in which measurement was made of the transport of a non-metabolizable sugar out of the cell. The technique was briefly as follows: the heart was first perfused with a high concentration of L-arabinose in order to accumulate a large amount inside the cell. The medium was then changed to sugar-free buffer which passed a single time through the heart and was collected over successive intervals of 1 or 2 minutes.

TABLE III

THE EFFECT OF INSULIN AND ALLOXAN DIABETES ON GLUCOSE TRANSPORT IN THE ISOLATED RAT HEART

The number of experiments is shown in parentheses. The asterisk denotes a value of  $<0.01$  with reference to the normal control without insulin.

Alloxan was injected where indicated in a dosage of 60 mg/kg i.v. 48 hours before sacrifice. Food was withheld during the last 24 hours and the terminal blood sugar concentrations were uniformly about 300 mg per cent.

Type of animal	Insulin mU/ml	Glucose out mg/g/h $\pm$ S.E.M.	Glucose space ul/g $\pm$ S.E.M.
Normal	0	4.3 $\pm$ 0.5 (10)	227 $\pm$ 9
Normal	$1 \times 10^{-3}$	9.6 $\pm$ 0.5 (15)	455 $\pm$ 14
Alloxan diabetic	0	0.7 $\pm$ 0.2 (11)	227 $\pm$ 9

P < .01 vs Normal Animal - No Insulin

The amount of pentose appearing in the perfusate under these conditions could be used to measure transport out of the cell provided extracellular arabinose was first removed. The collection of perfusate during the first 5 minutes was therefore discarded since this time was sufficient to remove all extracellular sugar as judged by the washout of radioactive sorbitol (see Fig. 4). The greater sensitivity of this method can probably be ascribed to the fact that transport under these conditions is essentially uni-directional since the continuous washing prevents accumulation of extracellular sugar and minimizes back transport into the cell.

In Fig. 5 some of the results obtained are shown. Hypophysectomy led to a marked rise in transport as compared to the normal animal.

Alloxan diabetes caused a marked depression but of insulin *in vitro* the rate could be restored to the high value of the normal. In the diabetic insulin increase in rate demonstrating that a very broad rate is under hormonal regulation. The effect of insulin from hypophysectomized animals was relatively

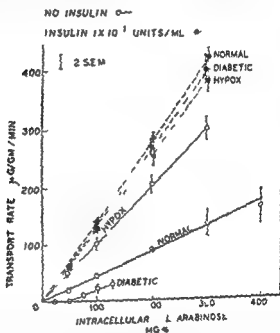


Fig. 5 The effect of alloxan diabetes and hypophysectomy on the transport of L-arabinose out of the cells of the isolated heart.

The technique is described in the text. The concentration of intracellular L-arabinose calculated by summing the pentose contents of the perfusate collections and the pentose of the tissue at the end of the experiment. The intracellular L-arabinose concentration was calculated on the assumption of a uniform distribution within the cell.

sumably to the already high control rate. Although the rates without insulin were widely separated in the three types of preparation, the rates with insulin were surprisingly close. It was also worthy that in any given series the rates showed remarkable constancy as judged by the standard error of the mean.

Fig. 6 shows the effect of various concentrations of insulin on

ing the preliminary period of pentose accumulation and continued during washout of the sugar  $1 \times 10^{-4}$  units per millilitre induced a large increase in rate amounting to 78 per cent of maximal represented by the value for  $1 \times 10^{-1}$  units/ml.  $1 \times 10^{-5}$  and  $1 \times 10^{-6}$  units/ml did not significantly stimulate the rate under the conditions tested. This sensitivity appears to be about the same as tested in other preparations such as the isolated rat diaphragm.

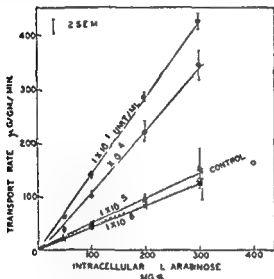


Fig. 6. The effect of intracellular concentration on the rate of outward transport of L-arabinose. The procedure is outlined in the text. The standard errors for the control rate are shown in parentheses.

The outward transport technique has also made it possible to determine with some precision the time of onset of insulin activity and follow the course of acceleration minute by minute. As seen in Fig. 7 when insulin was added to the perfusate during outward transport of sugar, an effect was noticed within about 2 minutes. The onset was equally quick in the diabetic muscle, but the subsequent acceleration was much slower. Even though the insulin added was 1000 times the minimal effective concentration, it took 10 to 40 times longer in the diabetic to reach the maximum rate.



This is a manifestation of the insulin resistance which has been frequently noted in muscle from alloxan-diabetic animals

It is of course most important to know what hormonal factors are responsible for the inhibition of transport in the diabetic state. On the basis of a large background of knowledge from many sources it would be anticipated that growth hormone and adrenal steroids might be involved. Fig. 8 shows the effect on transport of

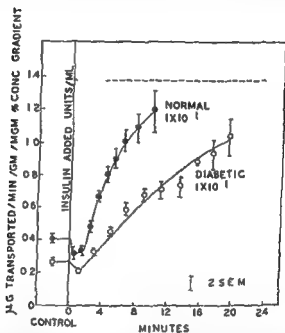


FIG. 7 The time course of the insulin effect in hearts from normal and alloxan-diabetic rats. Insulin was added in a concentration of  $0.1$  units per ml at 0 time. The horizontal broken line indicates the maximum rate obtained with insulin as determined in the experiments of Fig. 5.

daily injections of growth hormone for 5 days and/or cortisone into hypophysectomized animals. The rate was moderately reduced by either growth hormone or hydrocortisone alone and the combination of both hormones caused a severe depression of transport to the diabetic level. The observations suggest that a part of the well-known diabetogenic activity of growth hormone and cortisone may be attributed to inhibition of glucose transport in muscle. It should be noted however in the case of cortisone alone that very high

ses were employed. It remains to determine the effect of cortisone at lower levels since earlier studies of glucose utilization by muscle in hypophysectomized animals (Park *et al.*<sup>10</sup>) have not shown renal steroids to be effective inhibitors in the absence of the pituitary. In regard to growth hormone we have been unable to duplicate the interesting report of Bronk and Fisher<sup>3</sup> despite re-

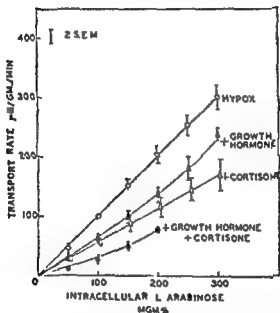


FIG. 8. The effect of growth hormone and cortisone on the transport of L-arabinose in the isolated hearts from hypophysectomized rats. Highly purified growth hormone gift of the Endocrinology Study Section of the National Institutes of Health was injected daily in dosage of 0.1 mg. per 100 g. per day. The daily dosage of cortisone was about 2.5 mg. per 100 g. per day. Treatment was continued 45 days. The heart injections were made about 24 hours before sacrifice.

peated efforts that growth hormone added *in vitro* inhibits glucose transport and utilization. Experiments carried out by Dr. Margaret Henderson in this laboratory using a very active growth-hormone preparation and also the same material H 163 employed by Bronk and Fisher have shown slight acceleration of transport in hearts from normal animals, and a large insulin-like acceleratory effect in hearts from hypophysectomized animals.

## CONCLUDING REMARKS AND SUMMARY

The regulation of transport appears to be the closest approach that we can make at present to defining the site of action of the above hormones on glucose uptake by muscle. It would not be proper however to assume that transport is necessarily the primary point of action of insulin or the other factors. The recent studies of Randle and Smith<sup>11</sup> for example illustrate that sugar permeability can be strongly influenced by changes in intracellular metabolism and a number of insulin effects on amino-acid and acetate metabolism discussed in this symposium and elsewhere are not explained at present by any single point of action.

The principal conclusions which we would like to draw from the present position of our work are as follows: the transport of D-glucose, D-3-O-methyl glucose, L-arabinose and D-xylose through the cell membrane in the presence or absence of insulin involves interaction of the sugar with a specific component of the membrane. The membrane-combining site shows substrate stereo-specificity and is shared by pentoses and hexoses. Glucose and 3-O-methyl glucose have high affinities relative to arabinose and xylose. The membrane-transport process can be regarded as the first step in the metabolism of sugar by muscle: it antecedes and is distinct from phosphorylation by the hexokinase system and the product of transport is the free sugar. In muscle transport is ordinarily slow and appears to limit the rate of glucose uptake. As the rate-limiting process it is an appropriate site for hormonal regulation. Insulin causes a striking acceleration of transport and alloxan diabetes a marked inhibition. The inhibition appears to be due to the effects of pituitary growth and adrenal steroids in conjunction with a lack of insulin.

Subsequent experiments using hearts from hypophysectomized rats have shown that the transport rates vary from those reported here to rates equal to or less than those of normal animals. The explanation of this variation is uncertain but may be related to variations in endogenous insulin levels to which the animals are unusually sensitive. Several mechanisms have been considered by which the *in vivo* injection of growth hormone and cortisone could result in lower transport rates. The hormones could (1) directly inhibit transport or (2) decrease the sensitivity of the tissue to endogenous insulin. At present we would favour the second

possibility. For additional discussion of these questions see Morgan H. E., Henderson M. J., Regen, D. M. and Park, C. R. *Ann NY Acad Sci* ■ 387 1959

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# INSULIN AND THE TRANSPORT OF SUGARS

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The perfused heart preparation has given insight into some of the problems associated with the permeability hypothesis of insulin action which are not easily attacked by the use of the whole or the eviscerated animal or by the use of the isolated diaphragm. This paper deals with some of these problems.

The perfused preparation is the most reliable one available for the investigation of the insulin-dependence of the penetration of sugar into cells. As Lindsay and I have shown (Fisher and Lindsay<sup>1</sup>), the penetration of galactose into the heart is markedly depressed in the presence of glucose in the perfusate. This is undoubtedly because both sugars are competing for a common carrier mechanism for the penetration of sugars with a lower affinity than glucose. A carrier would be expected to be correspondingly depressed by glucose. As a consequence when the sugar to be studied is introduced into the perfusate which contains glucose the rate at which the sugar enters the cells will not be a true measure of its intrinsic permeability but will be much more an index of its affinity for the common carrier. Furthermore, the intracellular glucose concentration it will increase, which glucose can compete for the carrier. This may obscure any insulin effect on the rate of penetration of the sugar. The scale of the insulin effect could be so small that the factors that it might be too small to be demonstrated.

We have made one test of these rather than the test of Sacks and Bakshi<sup>2</sup> showed that insulin increases the penetration into the rat heart in 10-15 minutes but has no effect on the penetration of D-arabinose. Dr Zachariah has shown that in the perfused rat heart in my laboratory that in a glucose-free perfusate L-arabinose penetrates into the cells as fast as galactose. The intracellular concentration of L-arabinose at an external concentration in 10-15 minutes is 10-15% of the external concentration.

already been shown by Sacks to be increased by insulin. D-arabinose penetrates much more slowly the intracellular concentration reaches only about one-third of the extracellular concentration after an hour. But the rate of penetration of D-arabinose is markedly increased by insulin. We hope to make competition experiments in order to determine whether the discrepancy between our finding and that of Sacks is due to the sort of factors which I have discussed.

One might perhaps have made this sort of test with the isolated diaphragm as indeed some workers have done but there are grounds for believing that the diaphragm does not yield useful indications of the kinetics of penetration. This is illustrated by our experience of the study of the kinetics of efflux of galactose from the heart in experiments of the same general nature as those described by Dr Park. Mr D. A. B. Young has made experiments in which the heart is first perfused with galactose and any desired concentration of insulin and is then transferred to a second apparatus in which the perfusate contains the same concentration of insulin but no galactose. After washing out the dead space effluent from the heart is collected in timed periods. By virtue of the high rate of perfusion the galactose concentration in the effluent is always very low so that we are effectively studying the efflux of galactose into galactose-free medium.

The data do not conform to a first order process. When the appropriate semilogarithmic plot is made the lines which are obtained always make positive intercepts on the ordinate axis. The data do however conform with one qualification to the much more complex equation for efflux from cylinders within which there is a uniformly low diffusion coefficient. The expected relation which is given by Crank<sup>5</sup> is shown in Fig. 1. In this figure the fraction of the intracellular solute extracted in time  $t$  is plotted against  $Dt/a^2$  where  $a$  is the radius of the cylinder and  $D$  is the diffusion coefficient within it.

We have transformed our observed fractions into the corresponding values of  $Dt/a^2$  with the help of this curve and tested the fit to the curve by plotting these derived values of  $Dt/a^2$  against time. We should expect straight lines through the origin provided that the fluid which we were collecting came immediately from the surfaces of the cells. Since exchange between interstitial fluid and perfusate is interposed between the cell surface and our collecting vessels we can expect the experimental curve to deviate to some

extent from a straight line through the origin. We have made a theoretical study of the effect of superimposing an exponential exchange between tissue fluid and perfusate on the Crank relation. We find that if the time-constant of the exponential process is  $1/k$  then the plot obtained should be a straight line for all times greater than  $3/4k$  and that this line should meet the time-axis at  $t = 1/k$ . This theoretical expectation is shown in Fig. 2. Our experimental data fit well with this as is shown in Fig. 3. Data obtained without

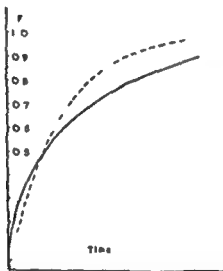


FIG. 1. The expected time-course of equilibration of an insular volume of solution with a cylinder in which the diffusion coefficient is uniformly low. The interrupted curve represents first-order equilibrium in such that the time for 50 per cent equilibration is the same as the time for 50 per cent equilibration of the diffusion through the cylinder.

insulin and with a wide range of insulin concentrations is shown in this figure and it will be seen that despite a great variation in the slopes of the lines they cut the time-axis in general in the region of 0.5 minutes. The first points on the lines are therefore on the linear portions of the curves and we can interpret the slopes of these lines as measures of  $D/a^2$ .

The intercepts on the time-axis indicate a half time of exchange between tissue fluid and perfusate of 0.3-0.4 minutes. Despite this



already been shown by Sacks to be increased by insulin. D-arabinose penetrates much more slowly; the intracellular concentration reaches only about one-third of the extracellular concentration after an hour. But the rate of penetration of D-arabinose is markedly increased by insulin. We hope to make competition experiments in order to determine whether the discrepancy between our finding and that of Sacks is due to the sort of factors which I have discussed.

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sufficient exchange between cells and medium in the perfused heart the kinetics of cell penetration are still obscured to some extent by extracellular exchange processes. This sort of obscuration must be much more marked in the isolated diaphragm. Not only is the time-course of exchange between medium and extracellular fluid much slower but the cells of the tissue must be non-uniformly exposed to the sugar being studied for a large part of the period of observation. Judging from Lippman and Cori's<sup>2</sup> data for thiosulphate the extracellular concentration of solutes entering the diaphragm from the external medium is not likely to become stable until more than 30 minutes have passed, even for small molecules. With something of the size of insulin the delay may be expected to be much longer. Probably therefore one is dealing over a large fraction of the period of observation with a preparation in which the cells are not only non-uniformly exposed to the sugar but even more non-uniformly exposed to insulin.

Reverting to our data for the efflux of galactose from the heart we can now consider the significance of the values of  $D/a^2$  which we obtain from the slopes of the straight lines in Fig. 3. Since  $a$  is the radius of the heart cell which can be assumed to be constant these variations imply variations in  $D$  the apparent diffusion coefficient of galactose in the cell. This apparent diffusion coefficient will be the product of the true diffusion coefficient of the galactose-carrier complex and of the ratio of galactose-carrier complex concentration to free galactose concentration. Since the diffusion coefficient of the complex is unlikely to be variable the variation in apparent diffusion coefficient may be taken as a measure of the variation in concentration of total available carrier. We have therefore made some preliminary explorations of theories of insulin action on cell permeability on the assumption that the concentration of available carrier is altered by insulin and is directly proportional to the experimentally determined value of  $D/a^2$ .

In the first place we have tested the hypothesis suggested by Fisher and Lindsay<sup>8</sup> that the carrier (C) combines with an inhibitor (X) to form an inactive complex (CX) and that insulin also combines with (X) to form a complex (IX) thus freeing carrier. This leads to an equation with four unknowns. We have fitted data for controls and for four insulin concentrations to this hypothesis by the method of least squares and found that the resulting equation fits all the points remarkably well. This is shown in Fig. 4.

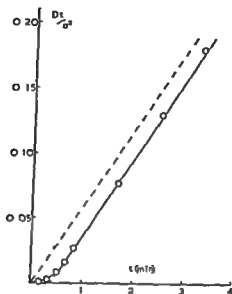


FIG. 2 The effect of interposition of an exponential exchange between cells and perfusate on the plot of  $Dt/a^2$  against  $t$

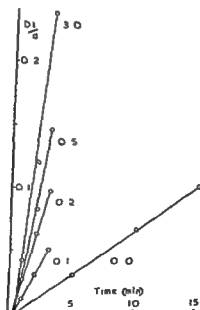


FIG. 3 The experimentally determined relation between  $Dt/a^2$  and  $t$ . The numbers against the lines represent the concentrations of insulin used in unit/litre

The presence of insulin is a measure of the insulin-dependent carrier. We have also supposed that the insulin-dependent carrier arises by combination of insulin with some cell constituent present in limited amount so that there is a Michaelis-Menten type of relation between insulin concentration and increase in apparent diffusion coefficient over the control value. This hypothesis is easy to fit to the data by standard methods and as Fig. 5 shows it fits just as well as the first hypothesis. The two best fit curves shown in Figs. 4 and 5 are scarcely distinguishable.

It ought however to be possible to distinguish between the hypotheses since one involves affinity of the sugar studied for a single carrier and the other its affinity for two separate carriers and it is highly unlikely that the affinities for two independent carriers of two or more different sugars will run in parallel. Repetition of the work with other sugars is therefore planned. Another approach suggested by the work of Bronk and Fisher<sup>2</sup> on the effect of growth hormone preparations on the permeability of the heart to galactose. This work suggested that a purified beef growth hormone preparation approximately halved the apparent diffusion coefficient of galactose into the heart both in the presence of and in the absence of insulin. This is much more easily explained on the single carrier hypothesis.

At present we incline towards the single carrier hypothesis, since the dual carrier hypothesis is not needed to account for penetration in the absence of insulin and since the growth hormone observations are more readily accounted for by the single carrier hypothesis.

Work of this kind cannot naturally contribute much to the important question of the ability of the permeability effect of insulin to account for all its physiological activity. But very few of its effects have been claimed to be totally inexplicable as secondary consequences of the permeability effect. The latest of these is the effect on the carbohydrate metabolic pattern of the isolated diaphragm which has been demonstrated so elegantly by Chain *et al.*<sup>4</sup> We would like to conclude by offering an explanation of this effect in terms of the permeability effect.

It is clear from the work of Candella *et al.*<sup>3</sup> Zierler *et al.*<sup>10</sup> and Zierler<sup>11</sup> that enzymes are continually escaping from the cells of the usual kind of diaphragm preparation. Escape will certainly occur from damaged cells but Zierler believes that he has evidence that most of this loss occurs across the intact cell membrane. If this is so

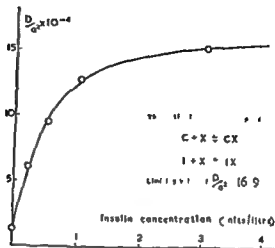


FIG. 4 The fit of the experimental data to the single carrier hypothesis of insulin action.

We have also tested an alternative hypothesis namely that there are two carriers one insulin-independent and one insulin-dependent as suggested by Resnick and Hechter<sup>8</sup>. To do this we have supposed that the apparent diffusion coefficient in the absence of insulin is a measure of the activity of the insulin-independent carrier and that the difference between this and the apparent diffusion coefficient in

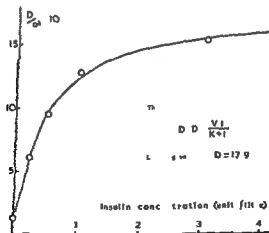


FIG. 5 The fit of the experimental data to the dual carrier hypothesis of insulin action



it will be evident from what has been said about diffusion in the diaphragm that enzyme concentrations in the interstitial fluid will much exceed those in the medium. It is therefore well within the bounds of possibility that the interstitial fluid may constitute a quite active kind of Meyerhof muscle extract. (One would expect that the enzymes most readily lost from muscle cells would constitute a glycolytic system in view of the known association of oxidative systems with the particulate fraction of the cell.) In the absence of insulin when the extracellular glucose concentration should be much higher than the intracellular concentration this would mean that glucose metabolism would be primarily glycolytic though of course the pattern would not necessarily mimic exactly that of glycolysis in muscle cells. In the presence of insulin when glucose can pass readily into cells the pattern would be expected to shift towards oxidative and anabolic pathways. Some support for this is to be found in earlier data of Beloff-Chain *et al*<sup>1</sup> which suggest that in the absence of insulin most of the glucose in the diaphragm is extracellular.

I believe therefore that there is as yet no need to move from the position that the permeability effect of insulin is in all probability responsible for its physiological effects.

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#### DISCUSSION

YOUNG The earlier studies of Hober, Loewi, Pollak and others have indeed fallen on most fruitful ground in recent years and I think Dr Park's contribution is a most interesting and important one in this connection. It particularly appeals to me that he is able so elegantly to make use of

the outward transport of sugars from cells in order to study the various hormonal effects

I would like to ask Dr Park a question myself — you mention in your abstract that you have found either no effect of growth hormone *in vitro* on glucose transport or an acceleratory effect. Do you find this acceleratory effect in hearts from normal rats or in hearts from hypophysectomized rats, or in both sorts of heart?

LARK: In the heart from normal rats we have not observed any significant effect of growth hormone *in vitro* on transport. In hearts from hypophysectomized rats there is a marked acceleratory effect.

LEVINT: In connection with Dr Lark's experiments on outward transport I should like to tell him that Dr Goldstein has confirmed his findings using the eviscerated nephrectomized dog. This phenomenon which Dr Lark demonstrated in the perfused heart obtains in the whole eviscerated animal which must include of course skeletal muscle and other insulin-sensitive tissues.

Some years ago Dr Goldstein and I injected cortisone into intact dogs in high amounts until the animals became insulin insensitive. Then we eviscerated them and studied the distribution of galactose with and without insulin. We could not, and cannot at present demonstrate any inhibition of insulin action in the eviscerated animal. I am wondering therefore whether we did not use a sufficient amount of cortisone to demonstrate an inhibition or whether the inhibitory effect is not present in all tissues. In the whole animal where the mass of the heart of course is small in comparison with the muscle and fat tissues, the inhibition phenomenon may not become significant. This would be important to resolve. One could draw the conclusion that this inhibition phenomenon has a relation to the diabetogenic effect in the whole organism and it is not yet clear whether it does or not.

SMITH: I am sure we would all agree with Dr Fisher that there are limitations to the use of diaphragm in studies of the transport of sugars. There is however one point in his discussion on which I would like to comment.

In the work which Dr Randle and I describe in our own paper (see p. 64) we were indeed aware of reports that certain enzymes are believed to leak from the hemidiaphragm during incubation but we could not show that these would utilize free glucose. We incubated a number of hemidiaphragms in bicarbonate medium for 30 minutes under our usual conditions then with fresh portions of the medium and incubated these again for a further period of 1 hour and the same conditions but in the absence of tissue no further change of glucose content of the medium occurred after separation from the tissue. Undoubtedly protein is released into the medium by the isolated hemidiaphragm — mainly from



the cut ends of the fibres I imagine — and this material may well include certain of the enzymes concerned in the utilization of glucose. But it seems from this experiment that under our conditions at any rate no dissimulation of glucose itself occurs in the medium bathing the tissue. Of course we do appreciate that this does not exclude the possibility of some glucose being metabolized in the extracellular compartment of diaphragm by enzymes bound to the surface of the cell as Shaw and Stadie have recently suggested.

STEWART Keyl and Dragstedt have shown that the embryonic chick heart 3 days old is refractory to the toxic action of cardiac glycosides and that at the tenth day the heart becomes sensitive to the glycosides. It is at this later stage that the pancreas begins to function. We have found in the guinea-pig pre-treated with insulin that the toxicity of the glycosides digoxin, digitoxin and ouabain is markedly enhanced and I wonder whether Dr Park or Dr Fisher have done any work on sugars such as digitoxose to know whether the transport of them is markedly accelerated by insulin or whether any other sugars linked to other substances to form glycosides are also rapidly taken up.

FISHER I do not know whether Dr Park and I ought to come here as twins and say No together but certainly for myself I have not and it is a very interesting idea.

Whilst I am here I would just like to say something about this business of the growth-hormone effect. I think a possible explanation is that when we measured the effect of our Wilhelm beef growth-hormone preparations on the penetration of galactose into the heart we had to have glucose present in the perfusate to slow the penetration down to manageable dimensions and it is quite conceivable that the effect that we obtained there is secondary to the effect on glucose metabolism.

D ARCY I would like if I may to direct my question to Dr Park. I was very interested to hear Dr Park mention that hypophysectomy increases the transport rate whereas growth hormone and cortisone in vivo depresses it. I would like to know for information if you have tried the effects of corticotrophin either *in vivo* or *in vitro* preparations.

PARK We have not tested this at all.

D ARCY I have wondered if that may possibly in some way explain the difference between your *in vivo* effects with growth hormone and your opposite results which you found *in vitro*. Could it be contamination of your growth-hormone preparation say with another trophic hormone from the pituitary?

PARK Contamination by other insulin-like or hypoglycaemic factors or factors of insulin like action, a possibility and mixed with pituitary

0 or in 1

hormone preparations in many laboratories and efforts to separate this activity by chemical means from growth activity have not been successful in our hands.

**BESSMAN** I should like to ask whether the kinetics of sugar transport might be equally referable to an energy mechanism. Wouldn't the equations fit just as well? There may be an energy mechanism which insulin enhances or which insulin inhibits and it may not be necessary to refer all the thinking merely to a membrane—I apologize for the word merely to this very mysterious thing called a membrane. It might be more fruitful to compare the different acceptor systems for energy in the various tissues. We find the heart different from the diaphragm and also from the skeletal muscle. We also find all the tissues different from the brain and it is probable that the various energy transport mechanisms or energy-developing mechanisms differ in the various organs particularly in the anatomic arrangement of the enzymes involved.

We have proposed a hypothesis which assigns the function of insulin to the connection of hexokinase to the energy-generating site of mitochondria. Under these circumstances we might possibly say that the pools which were mentioned of different concentrations of glucose would well be different areas in the cell (as we can see on electron microscopy) with the membranes within the cell sequestering the mitochondria of an enzyme system were attracted to or attached to a mitochondrion rendering that area of the cell more effective—it would produce a different pool of utilization of sugar and generation of energy.

**KRAHL** Dr. Hewson Swift and I have electron micrographs of diaphragm which show a relatively thick layer of mesothelial tissue on both the pleural and peritoneal sides of the true diaphragm muscle. It may be that these layers are related to the differences in kinetics of entry of various sugars and to the differences between diaphragm and heart muscle in their responses to insulin.

**LEIBOWITZ** Just a very small point—it has been shown for example by Dr. Nicholls at King's, that the effect of cortisone on the reticulo-endothelial system is antagonized by oestrogens. We know that cortisone is diabetogenic if anything whereas oestrogens have the opposite effect in the Houssay animal and I wondered whether you had tried the effect of oestrogens in antagonizing the effect of the cortisone. The second point is that in discussing these effects in animals we have to remember that the adrenal corticoids do not start being produced normally in the animal immediately at birth—they only start being produced immediately afterwards, and that the nature of the hormones produced by each animal is different. For example the rat produces I think a lot more cortisone than cortisone so I think we have a very complicated picture there.

**PARK** We have done nothing at all with the oestrogens. I would say

the cut ends of the fibres I imagine — and this material may well include certain of the enzymes concerned in the utilization of glucose. But it seems from this experiment that under our conditions at any rate no dissimilation of glucose itself occurs in the medium bathing the tissue. Of course we do appreciate that this does not exclude the possibility of some glucose being metabolized in the extracellular compartment of diaphragm by enzymes bound to the surface of the cell as Shaw and Stadie have recently suggested.

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FISHER I do not know whether Dr Park and I ought to come here as twins and say No together but certainly for myself I have not and it is a very interesting idea.

Whilst I am here I would just like to say something about this business of the growth hormone effect. I think a possible explanation is that when we measured the effect of our Wilhelmu beef growth hormone preparations on the penetration of galactose into the heart we had to have glucose present in the perfusate to slow the penetration down to manageable dimensions and it is quite conceivable that the effect that we obtained there is secondary to the effect on glucose metabolism.

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PARK We have not tested this at all.

D ARCY I have wondered if that may possibly in some way explain the difference between your *in vivo* effects with growth hormone and your opposite results which you found *in vitro*. Could it be contamination of your growth-hormone preparation, say with another trophic hormone from the pituitary?

PARK Contamination by other factors is always a possibility and insulin-like or hypoglycaemic activity has been observed with pituitary factors other than growth hormone. On the other hand an immediate insulin-like activity *in vivo* or *in vitro* has been observed with growth-

PART III

INSULIN AND INTERMEDIARY METABOLISM

*Chairman* PROFESSOR F G YOUNG



# SOME OBSERVATIONS ON THE MODE OF ACTION OF INSULIN

E. B. CHAIN

*Istituto Superiore Di Sanità Rome*

The following is a brief summary of some of the work on the mode of action of insulin which has been carried out in this laboratory by the author and his colleagues over the last 8 years

As is well known Gemill<sup>1,2</sup> made the important observation that insulin stimulates glucose uptake and glycogen synthesis in the isolated rat diaphragm. This observation was confirmed by many authors among them Villee and Hastings<sup>13</sup> who were the first to use U-<sup>14</sup>C-labelled glucose and followed the fate of the <sup>14</sup>C carbon in various fractions. Their results summarized in Table I formed the starting point for the present investigations.

As will be seen from the figures in Table I while in confirmation of Gemill's results there was an increase in glucose uptake and glycogen synthesis the larger part of the glucose disappearing from the incubation fluid could not be accounted for either in the absence or in the presence of insulin and furthermore the increased synthesis of glycogen in the presence of insulin was insufficient to account for all the extra glucose disappearing from the incubation medium. The author and his colleagues therefore set themselves the task to get a quantitative balance sheet of all the metabolites formed from glucose in the rat diaphragm in the presence and absence of insulin with the view of ascertaining the fate of the extra glucose metabolized in the presence of insulin.

## SEPARATION AND QUANTITATIVE DETERMINATION OF RADIOACTIVE METABOLITES BY PAPER CHROMATOGRAPHY

Over 90 per cent of the radioactive metabolites formed from glucose were recovered in an aqueous alcoholic extract of the homogenized muscle and in the medium of incubation. A small part shown to be glycogen remained in the insoluble residue.

The metabolites in the incubation medium and the muscle extracts were separated by paper chromatography and quantitatively deter-

mined by a scanning device. In the initial stages of these investigations an apparatus similar to that of Tomarelli and Florey<sup>14</sup> was used in which a paper strip is passed by hand under the window of a Geiger counter and its radioactivity is measured per unit area. This was an extremely laborious procedure and can only be used for monodimensional chromatography. To achieve a sufficient degree of separation of the metabolites it became necessary to use bidimen-

TABLE I

IN VITRO UTILIZATION OF <sup>14</sup>C LABELLED GLUCOSE BY RAT DIAPHRAGM MUSCLE

	Normal		Diabetic	
	Without insulin	With insulin	Without insulin	With insulin
Number of animals	14	14	7	7
1 Total glucose disappearing from medium * mg g per hr	1.53 ± 0.046	2.87 ± 0.047	0.78 ± 0.035	2.17 ± 0.10
2 Glucose metabolized to CO <sub>2</sub> * mg g per hr	0.11 ± 0.022	0.22 ± 0.036	0.08 ± 0.019	0.11 ± 0.030
3 Glycogen synthesized* mg g per hr	0.17 ± 0.062	0.62 ± 0.13	0.14 ± 0.045	0.61 ± 0.19
4 Glucose unaccounted for mg g per hr	1.25	2.03	0.56	1.45
5 Utilized glucose recovered as CO <sub>2</sub> per cent	7.2	7.7	10.3	5.1
6 Utilized glucose converted to glycogen per cent	12.1	21.6	18.0	28.1
7 CO <sub>2</sub> in centze well derived from glucose in medium per cent	10.4	16.3	8.1	9.2

\* The values given are means ± standard error  
After Valle and Hastings

sional chromatography and it was therefore essential to develop automatic scanning devices. Due to the limited time I shall not enter into the details of this work which has been published in full.<sup>15</sup> A number of models were developed and one now in routine use is illustrated in Fig. 1. The paper chromatogram is held in a frame which passes across two end window Geiger counters discontinuously from position to position thus exposing for counting a certain area of a certain size determined by the size of diaphragms.

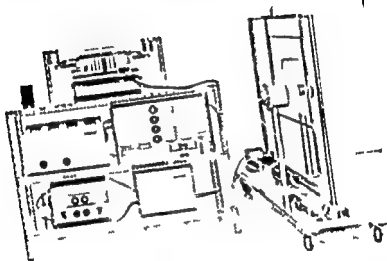


Figure 1. A schematic diagram of a mechanical system, showing a pump or engine component with various internal parts and a large, curved, ribbed section on the right side.



PLATE 22

CHAIN



FIG. Number 1 p and cor e ponds e rad aut gram

cross the end windows for a certain time period. The frame is moved first horizontally then when one full line is explored, back to the original position and vertically downwards to explore the next line. The time period depends on the intensity of the radiation emanating from the scanned area of the radiochromatogram when there is only background radioactivity the scanning period is 45 seconds; if the background threshold is exceeded the paper is exposed five times longer. The movements of the paper and the timing operations are controlled electronically; there are no gears or electromechanical parts.

The recording device for the Geiger pulses consists of an electric typewriter the number keys and return key of which are actuated by a series of solenoids. An electronic discriminator transmits the number of pulses collected by the Geiger counters per time unit and unit area to the appropriate solenoids on the number keys and writes it on a blank sheet of paper which is moved by the carriage of the typewriter in synchronization with the radiochromatogram. Number maps are thus obtained which give the position of the radioactive spots and their intensity in number of counts. A typical number map and its corresponding radiochromatogram are shown in Fig. 2.

This model has been further simplified by attaching the Geiger counters directly to the back of the typewriter and using the carriage of the typewriter for moving the radiochromatogram which is stuck with scotch tape to one end of the blank paper sheet inserted in the typewriter carriage (Fig. 3); thus the frame moving the paper chromatogram is eliminated.

A simple electronic scanner of monodimensional chromatograms based on a similar principle has also been developed (Fig. 4). It has the great advantage over the ratemeter in that it gives the number of counts per unit area scanned from background up to any value. A typical monodimensional number map is shown in Fig. 5.

#### INFLUENCE OF INSULIN ON GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM

The metabolites formed from  $U^{14}C$  labelled glucose in the extract of rat diaphragm and in the incubation medium are shown in the monodimensional radiochromatograms shown in Fig. 6. They have been identified as lactate, 2-phosphoglycerate (2-phog), malate, (m), 2-ketoglutarate and glycerate (g). A better separation of the

CHAIN

PLATE 2



FIG. Number in p and or c ponding rad o utog an

across the end windows for a certain time period. The frame is moved first horizontally then when one full line is explored, back to the original position and vertically downwards to explore the next line. The time period depends on the intensity of the radiation emanating from the scanned area of the radiochromatogram when there is only background radioactivity the scanning period is 45 seconds; if the background threshold is exceeded the paper is exposed five times longer. The movements of the paper and the timing operations are controlled electronically; there are no gears or electromechanical parts.

The recording device for the Geiger pulses consists of an electric typewriter the number keys and return key of which are actuated by a series of solenoids. An electronic discriminator transmits the number of pulses collected by the Geiger counters per time unit and unit area to the appropriate solenoids on the number keys and writes it on a blank sheet of paper which is moved by the carriage of the typewriter in synchronization with the radiochromatogram.

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#### INFLUENCE OF INSULIN ON GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM

The metabolites formed from U-<sup>14</sup>C-labelled glucose in the extract of rat diaphragm and in the incubation medium are shown in the monodimensional radiochromatograms shown in Fig. 6. They have been identified as lactate,<sup>1</sup> phosphate esters (in zone g), maltose (m), oligosaccharides and glycogen (ps). A better separation of the

metabolites is achieved by bidimensional chromatography (Fig 7)

The insulin effect on these metabolites is apparent without any quantitative analysis from a visual inspection of the radiochromatogram given in Fig 8 Glycogen the oligosaccharides and maltose are increased in the presence of insulin while there is no

N  
68  
61  
59  
72  
57  
62  
257  
815  
2148  
723  
201  
54  
59  
62  
54  
63  
68  
59

FIG 5 Part of map of a monodimensional radiochromatogram

apparent effect on the lactic acid phosphate esters or the glucose present in the tissue The results of the quantitative evaluation of the radiochromatograms by the scanning methods outlined above and the determination of radioactive  $\text{CO}_2$  formed are shown in Tables II and III They confirm the visual impression and in addition show that insulin has no effect on the production of radioactive  $\text{CO}_2$  Since Tables II and III were completed many more experiments using the same technique have been carried out always with the same results

The rapidity of the insulin effect on the polymerization of glucose is shown in Figs 9 and 10 A definite increase in glycogen oligosaccharide and maltose in the presence of insulin is shown already after 3 minutes incubation and is very pronounced at 7 and 15 minutes

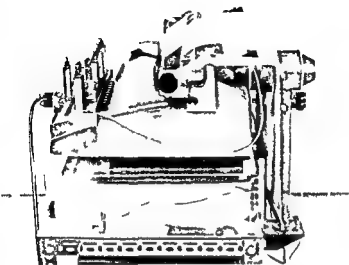
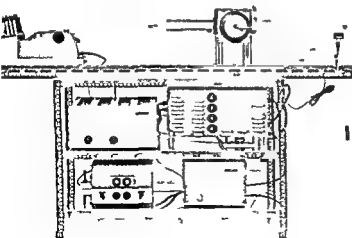


FIG 3 S d e f r g u t t e b d l d l r g p h y



F 4 S d f q t t e d e n o l r a d u h o a t o g p h y



FIG 6 Radiochromatogram of aqueous-ale 10% extract of red phage medium and medium before and after incubation with <sup>14</sup>C-labelled glutamate

A<sub>1</sub> = Medium before incubation  
A<sub>2</sub> = Medium after incubation



glucose

lactic acid

7 R d h to, of q co w-1 h l e tract of r t d ph gm m scl an  
 d w tl C l belled gl cose  
 d q l h r k m → b r l th of NH w r ↓ P cric acid tert ary  
 b tyl l oh l w ter





INFLUENCE OF INSULIN ON FRUCTOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM<sup>2</sup>

Fructose is much less metabolized than glucose by the isolated diaphragm muscle but the same metabolites are formed as are from glucose (Fig. 11). Quantitative evaluation of the radiochromato-

TABLE II

INFLUENCE OF INSULIN ON DISTRIBUTION OF <sup>14</sup>C IN GLUCOSE METABOLITES IN AN AQUEOUS-ALCOHOLIC EXTRACT OF RAT DIAPHRAGM MUSCLE AFTER INCUBATION WITH UNIFORMLY LABELLED GLUCOSE

Results expressed as counts (on p. per) per minute (c.p.m.)  $\times 10$  per 100 mg wet wt of diaphragm muscle. Insulin 5 units/ml. Glucose 0.5 p. cent. 10  $\mu$ C radio active glucose per Warburg vessel. 30-50 mg diaphragm per vessel in 6 ml. medium incubated for 90 minutes at 37°C in O<sub>2</sub>.

Ret	-ps Rf 0.003-0.07		-m Rf 0.16		-g Rf 0.30 Glu + ph phosphate ester			
	- Insulin      + Insulin		- Insulin      + Insulin		- Insulin      + Insulin		- Insulin      + Insulin	
1	29.5	51.8	—	—	26.1	27.2	Glu	
2	30.0	59.0	—	—	32.2	32.7		
3	19.1	55.0	6.0	10.2	43.7	47.4	PI phosphate ester	
4a	36.7	52.5	8.8	12.2	44.2	50.7		
4b	20.2	39.4	1.1	9.5	50.0	55.6	Insulin — +	
5	31.0	32.6	5.0	8.5	14.5	13.5		
6	24.5	38.0	6.1	11.6	17.0	18.5	7.4	9.7
Rf 0		Rf 0.03	Rf 0.07		Rf 0		Rf 0.03	
Insulin — +		Insulin — +	Insulin — +		Insulin — +		Insulin — +	
7	11.0	23.1	3.1	7.9	5.6	11.7	13.6	10.3
8	8.1	33.8	2.2	10.2	4.7	18.8	4.6	16.0

grams shows that insulin accelerates specifically the same reactions as those influenced in the case of glucose metabolism i.e. polymerization reactions leading to maltose oligosaccharides glycogen. There is again no effect on CO<sub>2</sub> production or lactate

formation nor is there any increase of the amount of free fructose in the tissue

# INFLUENCE OF EXTERNAL GLUCOSE CONCENTRATION ON THE PATTERN OF GLUCOSE METABOLISM IN THE ISOLATED RAT DIAPHRAGM IN RELATION TO THE PERMEABILITY THEORY OF THE MODE OF ACTION OF INSULIN

One of the widely current theories on the mode of action of insulin is the permeability theory of Levine and his colleagues<sup>10</sup>

TABLE III

INFLUENCE OF INSULIN ON DISTRIBUTION OF <sup>14</sup>C IN GLUCOSE METABOLITES AFTER INCUBATION OF RAT DIAPHRAGM MUSCLES WITH UNIFORMLY LABELLED GLUCOSE

Results expressed as (p.m.)  $\times 10^3$  per 100 mg wet wt of diaphragm muscle. Insulin 1 unit/ml. Glucose 0.1 per cent. 10  $\mu$ C radioactive glucose per Warburg vessel. 30-50 mg diaphragm per vessel in 6 ml medium, incubated for 90 minutes at 37°C in O<sub>2</sub>.

Rat	CO <sub>2</sub>		Lactate		Insoluble acids	
	- Insulin	+ Insulin	- Insulin	+ Insulin	- Insulin	+ Insulin
1	45.0	43.0	90.6	102.0	203.6	86.7
2	37.4	42.0	81.5	103.0	71.1	78.8
3	35.8	45.2	37.0	43.0	35.0	48.3
4a	30.4	28.4	48.0	38.4	—	—
4b	25.8	33.5	34.0	42.2	—	—
Glycogen insoluble residue						
						- Insulin + Insulin
5	14.4	12.8	56.8	88.2	2.5	43.5
6	18.0	18.4	34.8	36.6	56.8	24.4
7	24.2	18.0	—	—	8.3	26.0
8	36.0	28.4	—	—	11.9	15.1

According to this theory insulin acts by increasing the rate of entry of glucose into the cell. If this were the case it should be possible to simulate the specific effect of insulin on glucose metabolism by increasing the glucose concentration. Experiments to test this point were made by increasing the glucose in the incubation medium from 0.1 to 2 per cent and quantitatively determining by the scanning method referred to above the free glucose in the tissue and the metabolites formed from it.<sup>4</sup> The results are presented in Fig. 12

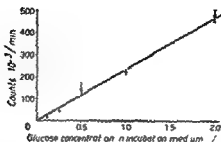


FIG. 12. Influence of the glucose concentration in the incubation medium on the intracellular concentration of the labeled diaphragm.

and Table IV. It will be seen that the free concentration of  $^{14}\text{C}$  in the tissue was proportional to the glucose concentration in the incubation medium. With increasing glucose concentration, radioactive CO<sub>2</sub> and the lactic acid increased considerably as pointed out above; this does not occur in the presence of insulin. The concentration of the oligosaccharides and glycogen also increased up to an external glucose concentration of 0.5 per cent, but above this concentration there was no further effect; the glucose polymerizing system was saturated at about 0.3 per cent. There was still a very marked specific insulin effect on malic acid, oligosaccharides and glycogen formation in the presence of as little as a concentration as 1 per cent glucose as shown in Fig. 13 and in the presence of 2 per cent glucose at these high glucose con-

TABLE IV

INFLUENCE OF GLUCOSE CONCENTRATION ON THE DISTRIBUTION OF  $^{14}\text{C}$  FROM UNIFORMLY LABELLED GLUCOSE IN RAT DIAPHRAGM MUSCLE

Results expressed c.p.m.  $\times 10^{-4}$  (counted on p. per) per 30 mg. tissue wet wt. Mean values of 15 experiments  $\pm$  S.E.M.

Glucose concentration in incubation medium (%)	0.1 per cent	0.25 per cent	0.5 per cent	2 per cent
Zone ps	105 $\pm$ 33	210 $\pm$ 47	410 $\pm$ 37	420 $\pm$ 203
Zone m	38 $\pm$ 0.6	95 $\pm$ 15	155 $\pm$ 30	170 $\pm$ 2.6
Zone k	126 $\pm$ 13	465 $\pm$ 25	220 $\pm$ 120	210 $\pm$ 215
Zone l	134 $\pm$ 10.4	2020 $\pm$ 163	255 $\pm$ 312	2750 $\pm$ 285
Glycogen (insoluble residue)	80 $\pm$ 23	140 $\pm$ 29	165 $\pm$ 50	170 $\pm$ 4.4
CO <sub>2</sub>	10.7 $\pm$ 8.0	24.0 $\pm$ 5.4	25.8 $\pm$ 7.7	19.5 $\pm$ 2.7

centrations insulin too showed no effect on  $\text{CO}_2$  and lactic-acid production. The effect of increasing the external concentration of glucose on the pattern of glucose metabolism in the isolated rat diaphragm is therefore qualitatively and quantitatively different from the specific effect of insulin. These experiments are therefore not in agreement with the permeability theory of the mode of action of insulin.

The main evidence for the permeability theory of the mode of action of insulin was based on experiments on the influence of insulin on the rate of galactose disappearance from the blood of eviscerated dogs<sup>10</sup> and later on similar experiments by Wick and Drury<sup>16</sup> with eviscerated rabbits and by Park and Johnson<sup>17</sup> with eviscerated rats. These authors found that insulin accelerated the rate of disappearance of galactose in the blood and on the assumption that galactose was not metabolized by muscle (the main remaining tissue in eviscerated animals) concluded that this effect was due to an increase in the permeability of the muscle cells to glucose. The premise on which these experiments were based i.e. that galactose is a metabolically inert sugar was shown to be incorrect by Wick and Drury in the eviscerated rabbit and by ourselves as well as Nakada and Wick<sup>11</sup> in the isolated rat diaphragm muscle using radioactive galactose. Radioactive  $\text{CO}_2$  was found to be formed though in small quantities demonstrating that muscle tissue contains the complete system for metabolizing galactose to  $\text{CO}_2$ .

The experimental findings on eviscerated animals are difficult to interpret in view of the fact that the animals were being continuously infused with glucose in order to counteract the hypoglycaemic effect of hepatectomy and insulin active glycogen and oligosaccharide synthesis was therefore occurring under these conditions. The effect of insulin on galactose uptake may therefore be of a secondary nature.

It would be more conclusive evidence if an effect of insulin on galactose metabolism could be demonstrated in the isolated rat diaphragm in the absence of glucose. Experiments to this end were carried out using radioactive galactose and the quantitative radio-chromatographic technique referred to above<sup>4</sup>. As shown in Fig. 14 the extract of rat diaphragm muscle incubated with galactose contained only galactose in measurable amounts. Small amounts of radioactive  $\text{CO}_2$  were also formed. Insulin had no effect on the concentration of free galactose in muscle diaphragm irrespective

of whether the muscle was incubated for 15 30 60 or (Table V)

#### THE UTILIZATION OF GLUCOSE BY RAT DIAPHRAGM MUSCLE UNDER ANAEROBIC CONDITIONS

Another theory of the mode of action of insulin which has been suggested has been that insulin accelerates the hexokinase reaction generally assumed to be the first step of glucose metabolism. Experiments on the effect of increasing glucose concentration on the pattern of glucose metabolism described in the preceding graph are incompatible not only with the permeability theory

TABLE V

EFFECT OF INSULIN ON THE INTRACELLULAR CONCENTRATION OF GALACTOSE-1 IN ISOLATED RAT DIAPHRAGM

Results expressed as c.p.m.  $\times 10^{-3}$  per 50 mg. tissue wet wt. Diaphragm incubated in  $O_2$  at 37° in 0.6 ml. phosphate-saline medium. Galactose concentration in medium of 0.1 per cent. Total radioactivity 1  $\mu$ Ci per Warburg vessel approximately  $7 \times 10^5$  c.p.m. (counted on paper). Number of experiments given in parentheses.

Time incubation min	Without insulin	With insulin
15	3310 $\pm$ 110 (6)	3340 $\pm$ 130
30	3420 $\pm$ 120 (6)	3370 $\pm$ 110
60	3540 $\pm$ 160 (4)	3280 $\pm$ 230
90	3030 $\pm$ 250 (8)	3490 $\pm$ 160

also with the hexokinase theory for if the action of insulin on the pattern of glucose metabolism cannot be simulated by increasing glucose concentrations it follows that it cannot be simulated either by increasing concentrations of the first product of glucose metabolism.

It was thought that if insulin acted on the hexokinase system such effect should be demonstrable also under anaerobic conditions after brief incubations in view of the fact that the hexokinase reaction is anaerobic provided sufficient ATP is present. To their surprise the present authors found that glucose is practically not metabolized by diaphragm muscle under anaerobic conditions<sup>1,6</sup>. As is apparent from Fig. 15 only free glucose is present in muscle incubated anaerobically with glucose for periods of 3 to 15 minutes in bicarbonate medium. In phosphate buffer for

metabolism of glucose is very reduced under anaerobic conditions, even after 60 minutes incubation very little radioactive lactate was formed (Table VI). However it was noticed that when diaphragm

TABLE VI

FORMATION OF LACTIC ACID FROM GLUCOSE BY RAT DIAPHRAGM MUSCLE INCUBATED UNDER AEROBIC AND ANAEROBIC CONDITIONS

Results expressed as c.p.m.  $\times 10^{-3}$  of lactic acid per 50 mg. tissue incubated in a phosphate-buffered medium containing 0.1 per cent glucose for 60 minutes. Radioactivity of uniformly labelled  $^{14}\text{C}$  glucose  $10 \mu\text{C } 7 \times 10^5$  c.p.m. Mean values given  $\pm$  S.E.M. Number of experiments given in parentheses.

Incubated in $\text{O}_2$	Incubated in $\text{N}_2$
$43.3 \pm 4.7$ (2)	$14.0 \pm 1.8$ (18)

muscle was incubated first in oxygen in the presence of glucose and then in nitrogen active lactic-acid formation took place but only for a few minutes. The presence of glucose was necessary in the

TABLE VII

INFLUENCE OF AEROBIC AND ANAEROBIC CONDITIONS ON LACTIC ACID FORMATION FROM GLUCOSE BY RAT DIAPHRAGM MUSCLE

Results expressed as c.p.m.  $\times 10^{-3}$  of lactic acid per 50 mg. tissue incubated in a bicarbonate-buffered medium containing 0.1 per cent glucose. Radioactivity per vessel  $10 \mu\text{C}$  of uniformly labelled glucose  $7 \times 10^5$  c.p.m. Mean value given  $\pm$  S.E.M. Number of experiments given in parentheses.

Conditions	*Incubated in $\text{O}_2$ 15 m n	*Incubated in $\text{N}_2$ 15 m n	*Incubated 15 m n $\text{O}_2$ 15 m n N introduced for 5 m n Incubated in $\text{N}_2$ 10 m n	Incubated 15 m n $\text{O}_2$ 15 m n N introduced for 5 m n *Incubated in $\text{N}_2$ 10 m n	†Incubated in $\text{O}_2$ for 15 m n $\text{N}_2$ introduced for 5 m n *Incubated in $\text{N}_2$ 10 m n
Lactic acid	$5.3 \pm 1.0$ (24)	$11.7 \pm 1.4$ (15)	$8.0 \pm 4.7$ (23)	$9.5 \pm 1.3$ (7)	$8.0 \pm 0.6$ (8)

\* Incubation in presence of  $^{14}\text{C}$  glucose

† Incubation in presence of non-radioactive glucose

aerobic phase radioactive glucose added in the anaerobic phase after previous exposure of the muscle to oxygen in the presence or absence of non-radioactive glucose was converted only in very small amounts to lactic acid (Table VII). In more recent experiments

It was found that it was sufficient to expose the rat diaphragm muscle to oxygen or to air for as short a period as 3 minutes in the presence of radioactive glucose to obtain the spurt of radioactive acid formation.

It seems thus clear that oxygen is an essential requisite for metabolism of glucose and no scheme which does not take account of this fact can be considered complete. That the failure of diaphragm muscle to metabolize glucose under anaerobic conditions is not simply due to a depletion of ATP is apparent from Table V.

TABLE VIII

INFLUENCE OF AEROBIC AND ANAEROBIC INCUBATION ON THE ADENOSINETRIPHOSPHATE CONCENTRATION OF RAT DIAPHRAGM MUSCLE

Results expressed as  $\mu$ mol of ATP per 100 mg. diaphragm muscle before incubation and after 3 minutes incubation in a phosphate buffered bicarbonate-buff red medium. Mean values of fifteen experiments  $\pm$  S.E.M.

Incubation	After incubation			
	In aerobically incubated		In anaerobically incubated	
	Aerob	Anaerob	Aerob	Anaerob
1230 $\pm$ 100	560 $\pm$ 63	470 $\pm$ 22	390 $\pm$ 64	630 $\pm$ 57

which shows that after 7 minutes incubation in nitrogen there is still sufficient ATP present to enable the hexokinase reaction to proceed. It should be recalled that under aerobic conditions there is a strong insulin effect on the polymerization of glucose after 7 minutes incubation.

The presence of ATP in rat diaphragm muscle after incubation under anaerobic conditions for 7 minutes could also be demonstrated using radioactive phosphate in the incubation medium though naturally its turnover rate was reduced in comparison with that occurring under aerobic conditions (Table IX).

It may altogether become necessary to revise the currently accepted idea that glucose-6-phosphate is the first intermediate from glucose in muscle. The experiment reported in Table X is suggestive in this direction.<sup>2</sup> In this experiment rat diaphragm muscle was first incubated with <sup>14</sup>C-glucose for 30 minutes and then after washing was incubated in the absence of glucose for varying time periods. Quantitative radiochromatography of the muscle extracts



## MECHANISM OF ACTION OF INSULIN

TABLE IX

THE INCORPORATION OF  $^{32}\text{P}$  INTO ADENOSINETRIPHOSPHATE IN RAT DIAPHRAGM MUSCLE INCUBATED UNDER AEROBIC AND ANAEROBIC CONDITIONS

Results expressed as c.p.m.  $\times 10^{-3}$  per 100 mg. tissue referred to an initial activity of the medium of  $5 \times 10^4$  c.p.m. (counted on paper) Diaphragm incubated in a phosphate-buffered medium containing 0.1 per cent glucose and 100  $\mu\text{C}$  of  $\text{Na}_2^{32}\text{PO}_4$ . Mean values given  $\pm$  s.e.m. Number of experiments given in parentheses

Time incubation min	Adenosinetriphosphate	
	Aerobic	Anaerobic
3	560 $\pm$ 32 (19)	303 $\pm$ 19 (12)
7	583 $\pm$ 23 (17)	241 $\pm$ 19 (8)
15	529 $\pm$ 30 (17)	92 $\pm$ 19 (8)

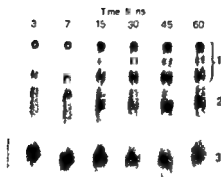
after different times of incubation showed that all radioactive polymerization products of glucose as well as the free glucose in the tissue disappeared rapidly, except the hexose phosphate esters which increased about four times (Table X). One would expect the first

TABLE X

DISTRIBUTION OF  $^{14}\text{C}$  AFTER INCUBATION WITH UNIFORMLY LABELLED GLUCOSE FOLLOWED BY INCUBATION IN THE ABSENCE OF SUBSTRATE

Results expressed as c.p.m.  $\times 10^{-3}$  per 50 mg. diaphragm muscle. Glucose initially present 0.1 per cent. Activity of medium c.p.m.  $\sim 7 \times 10^4$

Fractions		After incubation in the absence of substrate			
		After incubation with $^{14}\text{C}$ glucose	30 min	30 min	60 min
$\text{CO}_2$		12	30	51	102
Aqueous Ethanol Extract	Zone 1				
	Rf = 0 glycogen	137	50	45	16
	Rf = 0.03 oligosaccharide A	45	22	0.8	07
	Rf = 0.07 oligosaccharide B	110	31	29	27
	Zone 2				
	Maltose	85	25	23	25
	Zone 3				
	Rf = 0.30 glucose	111	59	22	27
	Rf = 0.34 hexose phosphates	34	185	224	133
Glycogen of insoluble residue		169	79	65	75



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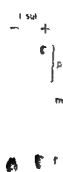
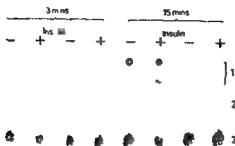


FIG. 11. Rad. chromatogram of aqueous-alcohol extract of rat diaphragm muscle incubated with uniformly labelled  $^{14}\text{C}$  fructose and with  $^{14}\text{C}$  insulin.

FIG. 13. Rad. chromatogram of aqueous-ethanol extract of rat diaphragm incubated for 90 minutes with a per cent glucose with and without insulin.

FIG. 14. Rad. autograph of chromatogram of aqueous-alcohol extract of rat diaphragm incubated for 30 minutes with 0.1 per cent galactose with and without insulin.



4

A B A B

Rad whr t l s f q e w l hol t r f r d pl r k n t u l e  
 d w th C l b l d g l u s e l j d i s i t e s i k r b e t d u i A e r b  
 t n B o b b e

## CHAIN



Fig. 17. R d i c h r i s t o g r a m t a q u u n f l l v t c r o t r d i l l r a g n m u c l  
u l a t e d w t h N a i O  
T y o d i e n o u l h r m a r g r a i p o p l a t i o n u m h y d r o x i w t e r f  
b u r v i c c d i n o i t n h y d r o d e

# E B CHAIN

product of glucose metabolism to be a very reactive substance not to accumulate in an actively metabolizing tissue as the phosphate was shown to do in this experiment

Experiments are in progress with radioactive phosphate with aim to ascertain whether any insulin effect could be shown on principle phosphorylated compounds formed in diaphragm muscle. The phosphorylated compounds were separated by chromatography (Fig 16) and quantitatively determined by the scanning referred to above. No effect of insulin on any of the compounds

TABLE XI

THE EFFECT OF INSULIN ON THE INCORPORATION OF  $^{32}$ P INTO ADENOSINETRIPHOSPHATE AND PHOSPHOCREATINE IN RAT DIAPHRAGM MUSCLE INCUBATED IN THE ABSENCE AND PRESENCE OF GLUCOSE

Results expressed as c.p.m.  $\times 10^{-4}$  per 100 mg of tissue incubated for 30 minutes referred to 80 mmol activity of the medium of  $50 \times 10^4$  c.p.m. Medium contained 100  $\mu$ Ci of  $N^{32}$  ATP

Experiments	Aden s n e t r i p h o s p h a t e				P h o s p h o c r e a t i n e			
	N o g l u c o s e		W i t h g l u c o s e		N o g l u c o s e		W i t h g l u c o s e	
	A t m o s p h e r e	I n t r a m u s c l e	A t m o s p h e r e	I n t r a m u s c l e	A t m o s p h e r e	I n t r a m u s c l e	A t m o s p h e r e	I n t r a m u s c l e
1	62.2	63.8	62.4	64.4	20.3	13.3	33.8	49.2
2	59.7	66.3	62.7	65.1	13.3	14.0	45.9	47.0
3	73.1	89.1	90.8	112.0	23.8	44.8	59.8	77.7
4	83.6	60.7	75.5	86.3	41.2	50.0	45.7	77.6
5	48.4	68.4	73.0	82.6	17.4	12.7	17.9	13.9
6	46.7	45.7	67.0	51.0	4.4	6.1	29.1	29.6
7	23.6	30.2	68.5	81.9	12.4	26.9	17.5	33.2
8	69.4	63.4	68.9	75.5	16.5	17.1	35.7	39.4
Mean	66.5 $\pm 5.4$	67.4 $\pm 4.6$	71.1 $\pm 3.2$	77.4 $\pm 6.5$	20.8 $\pm 3.7$	21.8 $\pm 5.0$	38.2 $\pm 4.4$	48.7 $\pm 7.6$

formed could be found except in some experiments but not in all a statistically significant effect on the turnover of phosphocreatine (Table XI). This effect occurred only in the presence of glucose. Experiments to determine the specific activities of phosphocreatine and ATP are in progress.

## EFFECT OF INSULIN ON GLUCOSE METABOLISM IN BROWN ADIPOSE TISSUE

In the brown adipose tissue of the rat glucose is converted into the same metabolites as it is in the rat diaphragm muscle i.e. malose

oligosaccharides glycogen and lactic acid (Fig 17) In is actively converted into fat Insulin was shown to cogen oligosaccharides and maltose synthesis from  $\beta'$  brown adipose tissue of the rat but had no effect on  $\text{CO}_2$  formation In fact it stimulated the same as it did in the rat diaphragm muscle In addition it notably the synthesis of fat from glucose (Table XII) T' brown adipose tissue of the rat insulin could be shown to two synthetic highly endergonic reactions

TABLE XII

INFLUENCE OF INSULIN ON GLYCOGEN AND FATTY ACID SYNTHESIS FROM  $^{14}\text{C}$  GLUCOSE IN RAT BROWN ADIPOSE TISSUE SLICES

Results expressed as c p m  $\times 10^{-3}$  per mg tissue wet wt Tissue incubated for 90 minutes in 0.6 ml phosphate medium at 37 in  $\text{O}_2$  Glucose concentration 0.1 per cent radioactivity 10  $\mu\text{C}$  per vessel  $7 \times 10^4$  c p m (counted on paper) Mean values of eleven experiments given  $\pm 1 \text{ c m}$

	No insulin	With insulin
Glycogen oligosaccharides and maltose (in aqueous alcoholic extract)	12 $\pm$ 01	33 $\pm$ 03
Fatty acids (in insoluble residue)	95 $\pm$ 25	177 $\pm$ 19

### CONCLUSIONS

The authors believe that none of the hitherto suggested hypothesis on the mode of action of insulin can account for all the experimental data Insulin appears to exert a specific stimulating effect on the energy-requiring synthetic reactions occurring in the cells on which it acts such as glycogen fat and protein synthesis It must act by making energy available for these reactions probably from a bound form by a mechanism which up to the present is not understood and forms the subject of present investigation in the authors laboratory

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# INSULIN ACTION AND THE PASTEUR EFFECT IN MUSCLE

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The uptake of glucose by many cells and tissues is known to be greater under anaerobic than under aerobic conditions. This stimulation of glucose metabolism in the absence of oxygen first recognized by Pasteur is at present thought to depend upon the fact that anaerobic metabolism of glucose yields a smaller amount of energy-rich phosphate than aerobic metabolism. In conformity with this view substances such as *m*-4-dinitrophenol which inhibit the production of energy rich phosphate during respiration increase the uptake of glucose by cells and tissues under aerobic conditions.

Insulin also increases the aerobic uptake of glucose by certain mammalian cells and tissues such as skeletal and cardiac muscle and adipose tissue leucocytes fibroblasts and lens. The mechanism of this effect of insulin on glucose uptake is not yet known though in the case of skeletal and cardiac muscle it is thought that the overall rate of uptake of glucose is limited by its rate of entry into the muscle cell and that insulin acts by accelerating this process (Levine and Goldstein<sup>1</sup> Park and Johnson<sup>2</sup> Park *et al*<sup>3</sup>).

This idea necessarily implies that any other factor which increases the uptake of glucose by muscle must also accelerate the entry of glucose into the cell. It was therefore important to know whether anoxia or substances which inhibit oxidative phosphorylation would increase the uptake of glucose by muscle in a similar manner to insulin i.e. by accelerating the entry of glucose into the muscle cell. We present here a comparison of the effects of insulin with those of anoxia and of substances which inhibit oxidative phosphorylation on the uptake of glucose and the intracellular accumulation of glucose and xylose by isolated rat diaphragm. A detailed account of most of the experimental methods used has been published elsewhere (Randle and Smith<sup>4</sup>).

## UPTAKE OF GLUCOSE BY ISOLATED RAT DIAPHRAGM

The uptake of glucose by isolated rat hemidiaphragm incubated

in a medium buffered with bicarbonate (measured as the change in glucose concentration in the medium during incubation for 1 hour at 37° C) was found to be increased by anoxia and by 2,4-dinitrophenol (0.05 mM or 0.25 mM), sodium arsenite (mM), sodium cyanide (mM) and sodium salicylate (5 mM) as well as by insulin (0.1 unit/ml). The uptakes ranged from about 150 per cent of the basal uptake in the presence of salicylate to about 400 per cent when cyanide or insulin were present (Fig. 1). On the other hand when diaphragm was incubated in a medium buffered with phosphate

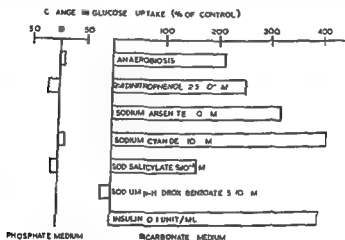


FIG. 1. The effect of insulin, anaerobiosis or some cell poisons on the uptake of glucose by isolated rat hemidiaphragm incubated in media buffered with phosphate or bicarbonate.

glucose uptake was not increased by anoxia, 2,4-dinitrophenol, sodium cyanide or sodium salicylate (Fig. 1) in conformity with the results of others who have used phosphate-buffered media (Demis and Rothstein<sup>1</sup>, Smith and Jeffery<sup>10</sup>, Villee *et al.*<sup>12</sup>, Walaas and Walaas<sup>13</sup>). The lack of effect of these factors on glucose uptake in phosphate medium appears to be due to inadequate buffering under these conditions (Randle and Smith<sup>6</sup>). We conclude therefore that the uptake of glucose by isolated diaphragm, like that of other cells and tissues, is increased by anoxia or by substances which inhibit oxidative phosphorylation provided adequately buffered media are used.

In order to determine whether anoxia and substances which inhibit oxidative phosphorylation might increase uptake of glucose by diaphragm by accelerating its entry into the muscle cell we have also investigated the effect of these factors on the accumulation of free D-glucose and D-xylose in isolated diaphragm.

#### ACCUMULATION OF GLUCOSE AND XYLOSE IN ISOLATED RAT DIAPHRAGM

The amount of a metabolizable sugar such as glucose which is present in the free form within a cell must depend upon the relative rates of its entry into the cell and of its further metabolism (see Fig. 2) In

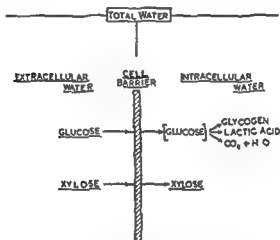


FIG. 2. Factors involved in the intracellular accumulation of free glucose and xylose in rat diaphragm.

diaphragm under normal conditions the latter exceeds the former and no free glucose accumulates (Park and Johnson<sup>6</sup>) although with variation of conditions the rate of entry may be enhanced or the rate of utilization depressed to such a point that the order is reversed and free glucose becomes detectable (Park *et al.*<sup>7</sup>) On the other hand this difficulty does not arise if a sugar such as xylose is used. D-xylose does not appear to be metabolized by diaphragm to any appreciable extent (Kipnis and Cori<sup>7</sup>) so that the rate at which it accumulates within the muscle cell may be expected to depend solely upon its rate of entry. Accordingly we have examined the

effects of insulin and of factors which inhibit oxidative phosphorylation on the intracellular accumulation of glucose and xylose in isolated rat diaphragm

In what follows the amount of free sugar which accumulates in diaphragm is expressed as a space the glucose or xylose space being that fraction of the volume of the tissue which contains fluid of the same specific gravity and glucose or xylose concentration as the incubation medium. Interpretation of these results in terms of the distribution of sugar between extracellular and intracellular fluids involves comparing the glucose or xylose space with the volume of extracellular fluid. Glucose or xylose is deemed to be present in intracellular water only if the glucose or xylose space exceeds the volume of extracellular water. The volume of extracellular water has been determined by measuring the space occupied by inulin, raffinose, sucrose or thiosulphate substances which are thought to be confined to extracellular fluid under normal conditions. We have found as others have that not all these substances yield the same value for the volume of extracellular fluid, the smallest value being obtained with inulin and the greatest with thiosulphate. Since it is not known which substance yields the most reliable estimate of extracellular fluid volume the conclusions which we draw are for the most part based on all estimates of the volume of extracellular fluid.

Spaces were measured in an intact diaphragm preparation (Kipnis and Cori<sup>2</sup>) in which none of the muscle fibres are cut. The advantage of this preparation is that its spaces are apparently similar to those of the muscle *in vivo*. Intact diaphragms were incubated singly in bicarbonate medium usually for 1 hour and the medium and tissue then analysed and the spaces calculated.

The distribution of free glucose between extracellular and intracellular water in isolated diaphragm under various conditions is shown in Fig. 3. It will be observed that when diaphragm was incubated in bicarbonate-buffered medium in the presence of oxygen free glucose appeared to be confined to extracellular fluid. This was still the situation when insulin, arsenite, cyanide or salicylate were present or oxygen excluded even though the uptake of glucose was greater in every instance (cf. Fig. 1). This must mean either that the potential rate of intracellular utilization of glucose under these conditions exceeds its rate of entry such that no free sugar accumulates or that glucose does not enter the muscle cell as the free sugar.

When diaphragm was incubated in the presence of 2,4-dinitrophenol however not only did the uptake of glucose increase (Fig. 1) but some free glucose accumulated in intracellular water (Fig. 3). Furthermore when sodium fluoride (which inhibits utilization of glucose) was present in addition to dinitrophenol even more glucose

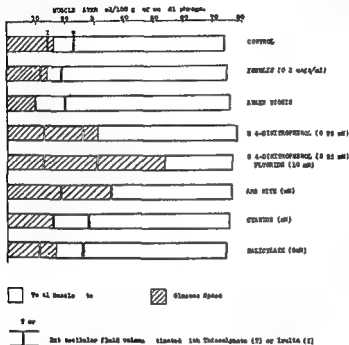


FIG. 3. The distribution of free glucose between intracellular and extracellular water in rat diaphragm incubated in bicarbonate medium. In the histogram the hatched areas on the left the limit of the muscle space on the right the limit of the extracellular space.

accumulated intracellularly. Hence unless the process of glucose uptake is abnormal when dinitrophenol and fluoride are present it must be concluded that glucose does indeed enter the muscle cell as the free sugar and that it accumulates only when the rate of utilization within the cell falls below the rate of entry. These results are therefore compatible with the view that it is the entry of



glucose into the muscle cell which is the rate-limiting step in glucose uptake and that insulin increases glucose uptake by accelerating the entry of glucose. Furthermore it would appear that anoxia and substances which inhibit oxidative phosphorylation likewise increase glucose uptake by accelerating its entry into the muscle cell.

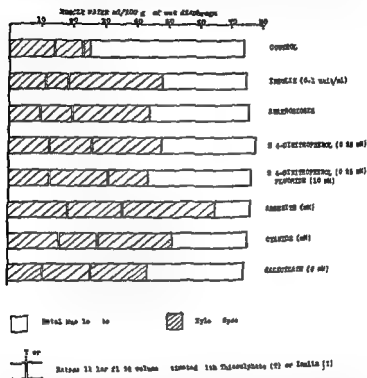


FIG. 4. The distribution of free xylose between extracellular and intracellular water in intact rat diaphragm incubated in bicarbonate medium. In each instance the breaks in the histogram are on the left the limit of the insulin space on the right the limit of the thiocyanate space.

Further support for this view is provided by the results of a similar series of experiments with D-xylose (Fig. 4). Under basal conditions a small amount of xylose accumulated in intracellular water (equivalent to equilibration with about 6-20 per cent of intracellular water) but in the presence of insulin, arsenite, cyanide, 2,4-dinitrophenol (with or without fluoride) or salicylate or in the

absence of oxygen the accumulation of xylose was greatly enhanced (equivalent now to equilibration with 40-60 per cent of intracellular water). We conclude that each of these factors also accelerates the rate of entry of xylose into the muscle cell. Although we do not yet know whether glucose enters the muscle cell by the same or a similar process to xylose it none the less seems reasonable to infer that these results lend support to the view that anoxia and substances which inhibit oxidative phosphorylation increase glucose uptake by accelerating the entry of the sugar into the muscle cell.

#### SPECIFICITY OF EFFECTS OF ANOXIA AND CELL POISONS ON PERMEABILITY

The investigations of Levine and his associates (see Levine and Goldstein<sup>23</sup>) and of Park and his colleagues suggest that the effect of insulin on the penetration of muscle cells by sugars has elements of specificity. For example sorbitol and mannitol although possessing physicochemical properties similar to those of glucose and other sugars do not enter the muscle cell either under basal conditions or in the presence of insulin. It is therefore pertinent to consider whether factors which inhibit oxidative phosphorylation accelerate the entry of sugars into the cells of diaphragm in a specific manner or whether they damage the cell barrier\* in such a way that substances leak through in a non-specific manner. We believe that the process is a specific one for the following reasons:

1. The rates of uptake of glucose and of xylose are always very different. The extra uptakes of glucose (mg of glucose/g of wet diaphragm/hr) ranged from 1.5 when salicylate was present to 6.4 with cyanide whereas the corresponding figures for xylose (mg of xylose/g of wet diaphragm/hr calculated directly from the xylose content of the tissue) were 0.45 and 0.55. If the accelerated entry of glucose and xylose was due to a breach in the cell barrier then we would expect the two sugars to enter the muscle at more comparable rates. Furthermore we would expect free glucose to accumulate within the muscle cell under these conditions.

2. With each of these factors the muscle cell still exhibits selective permeability. Thus in each case insulin, anoxia and cell poisons

\* The view that the entry of glucose into the cell is the rate-limiting process in its metabolism would seem to imply that the cell is surrounded by a limiting structure distinct from the major portion of the cell. While there is a considerable body of evidence for the existence of such a structure a consideration of this is not relevant to our present discussion and in order to avoid as far as possible a preconception as to its nature we will refer to it as the cell barrier.

promoted uptake of glucose and accumulation of xylose (Figs 1 and 3 and Table I) and also of fructose and ribose (unpublished observations) but not of inulin maltose raffinose sucrose or thio-sulphate (Table I) (The very small changes in the inulin maltose raffinose sucrose and thiosulphate spaces observed may be reasonably attributed to alterations in the volume of extracellular fluid) On the other hand if diaphragm was injured by cutting or by freezing with solid carbon dioxide extensive accumulation of inulin raffinose or thiosulphate as well as of glucose or xylose occurred (unpublished observations)

TABLE I

EFFECT OF INSULIN ANOXIA OR CELL POISONS ON SPACES OF RAT DIAPHRAGM

<i>Changes in spaces (ul / 100 mg of wet muscle)</i>						
	<i>Inulin</i>	<i>Maltose</i>	<i>Raffinose</i>	<i>Sucrose</i>	<i>Thiosulphate</i>	<i>Xylose</i>
Control	(14)	(16)	(20)	(21)	(23)	(26)
Insulin	-2	2	-4	0	-4	+2
Anaerobiosis	-4				-3	+18
2,4-Dinitrophenol	-1	+6	+6	+4	+3	+2
Cyanide	+5				+5	+6
Salicylate	-3	-1	0	-1	+3	+18

3 The enhanced rate of glucose uptake under anaerobic conditions was immediately reduced and the concurrent loss of potassium arrested by restoration of aerobic conditions during the course of the incubation (Fig 5) If the cell barrier had been irreversibly damaged during the anaerobic phase of the experiment it is to be expected that the loss of potassium and the more rapid uptake of glucose would have continued despite the readmission of oxygen

4 It was observed that insulin was still capable of increasing glucose uptake in the presence of 2,4-dinitrophenol arsenite cyanide or salicylate or in the absence of oxygen (Fig 6) As integrity of cell structure is believed to be essential for an effect of insulin it is not likely that it would have exerted an effect under anaerobic conditions or in the presence of these cell poisons if the cell barrier had been damaged by these agents

We conclude on the basis of these observations that factors which inhibit oxidative phosphorylation do not promote the entry of

sugars into the muscle cell by damaging the cell barrier. Our results suggest rather that like insulin they accelerate the entry of sugars by affecting the activity of a process which shows some degree of specificity.

#### REGULATION OF SUGAR ENTRY AND MECHANISM OF INSULIN ACTION

Although the details of the process whereby glucose enters the muscle cell are still obscure, experimental evidence appears increasingly to support the view that a transport process is involved. This may be envisaged as occurring in some manner such as that shown

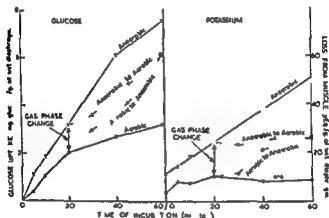


Fig. 5. The effect of anaerobiosis on the uptake of glucose and loss of potassium by isolated rat hemisaphagrum incubated in bicarbonate medium.

in diagrammatic form in Fig. 7. Glucose is depicted as combining with a specific constituent of the cell barrier at its outer surface. Thence it is conveyed in combination with such a carrier to the inner surface of the cell barrier, there to be released into the intracellular milieu. Since a number of factors which inhibit oxidative phosphorylation appear to be capable of accelerating the entry of glucose into the muscle cell, it seems reasonable to deduce that the activity of the putative transport system is in some way inhibited by energy-rich phosphate. One way in which this could occur is shown in Fig. 7. We suggest that the putative carrier may occur in

two interconvertible forms differing perhaps by a phosphate residue the phosphorylation occurring by reaction with an energy rich phosphate compound. The energy-rich phosphate compound is depicted here as adenosine triphosphate (ATP) but it does not have to be this particular energy-rich phosphate compound. If as we suggest only the non-phosphorylated form of the carrier is capable

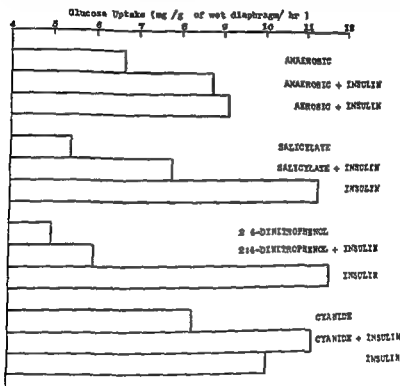


FIG. 6 Influence of anaerobiosis or cell poisons on the response to insulin of isolated rat hemidiaphragm incubated in bicarbonate medium

of combining with the sugar and of transporting it into the cell then our results would receive some explanation. Thus a deficiency of ATP induced for example by anaerobiosis (Ottaway<sup>5</sup>) would favour the non-phosphorylated form of the carrier and the transport of sugar into the cell.

Since insulin does not inhibit oxidative phosphorylation (Stadie<sup>11</sup>)

the mechanism of its effect on sugar transport is likely to be different from that suggested for anaerobiosis and substances which do inhibit oxidative phosphorylation. One possibility depicted in Fig. 7 is that insulin may increase the proportion of free carrier (and thereby accelerate transport of sugars) by interfering specifically with phosphorylation of the carrier in the cell barrier. Thus it might do either by inhibiting phosphorylation of the carrier or by stimulating dephosphorylation of the phosphorylated carrier.

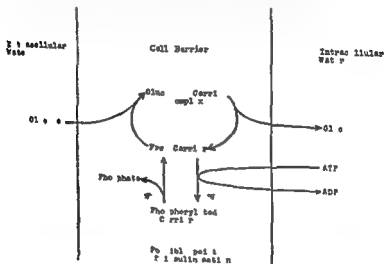


FIG. 7. A hypothesis for the mechanism of action of insulin.

This hypothesis of the regulation of entry of sugars into the muscle cell and of the mechanism of action of insulin is patently tentative. Alternative interpretations of the experimental observations are undoubtedly conceivable at the present time. Nevertheless the hypothesis outlined above does appear to have certain merits and advantages from the biological standpoint. It implies, for example, that the rate of entry of glucose into the muscle cell is geared directly to the energy requirements of the cell. It is also capable of providing some explanation for the increased uptake of glucose by skeletal muscle during contraction, for in contracting muscle much of the available ATP may be bound to the myofibril

and so not available to the process which we suggest is restricting the entry of glucose into the cell in the resting state. Finally it is of interest to note that the mechanism of action of insulin which we put forward has certain elements in common with those proposed on the one hand for the thyroid hormone which is believed to interfere with oxidative phosphorylation and on the other hand for glucagon which is thought to promote the formation of the active (in this case phosphorylated) form of liver phosphorylase.

The particular significance of the effects of sodium salicylate on glucose uptake by isolated diaphragm in relation to the use of salicylates in the treatment of diabetes has been discussed elsewhere (Manchester *et al* <sup>4</sup>).

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#### DISCUSSION

YOUNG Many investigators have perhaps thought of the possibility of designing a machine which would not only make measurements but which would also write up the results. Professor Chain and his colleagues have got nearer to this realization than most people. Certainly those of us who have had the privilege of seeing the results of his labours find them most impressive: the machines he uses are indeed astonishing.

LEVINE I was very much interested and intrigued by both of these papers. I should like to discuss them separately if I may.

Professor Chain in his previous publications and again now has drawn attention to oligosaccharides in muscle, presumably intermediates between glucose and glycogen. These observations are in accord with the finding by Dr Fishman in Boston of maltotriose and maltotetraose in liver.

Now in relation to the action of insulin directly Professor Chain makes the point that mere penetration or availability of glucose to the interior of the cell cannot explain the action of insulin since a high glucose level leads to an increase not only in oligosaccharides but also in the  $CO_2$

and lactic acid while if insulin is added he does not find an increase in the lactic acid or CO. This finding differs however from the results of many other workers so that we must note that many workers have found using labelled glucose that insulin does increase the yield of CO<sub>2</sub> and I will refer to the latest of these from Dr Renold and his group. Using the isolated fat pad the addition of insulin with uniformly marked glucose shows an increase in the yield of CO<sub>2</sub> and I believe Professor de Duve found similar results in other tissues (See also Vilcek using diaphragm *J Biol Chem* 1949).

The finding of an increased turnover of creatine phosphate in relation to insulin action is of interest as a datum but I should like to know from Professor Chain how he correlates this with the intense stimulation of glucose uptake, glycogen formation and fatty acid synthesis in adipose tissue which as far as I am aware does not contain creatine phosphate at all. In other words fat tissue which is very insulin sensitive does not contain the creatine phosphate mechanism and if that is supposedly the primary point of insulin action it fails to explain insulin action on fat tissues.

Coming now to Dr Smith's presentation of Dr Randle's paper it indicates that substances other than insulin may stimulate the transport system in the diaphragm or in other tissues for that matter. Some years ago we had shown that muscular exercise does so presumably by humoral mechanism and now it is shown that anaerobiosis and certain drugs increase the rate of a transport system which according to the studies here reported is specific for certain sugars of particular structure.

How are we to relate all these findings? With the permission of the Chairman who asked us to do so I should like to let my hair down. It will therefore be a mixture of fact and fancy. I hope not too much fancy. I believe that the effect of insulin in stimulating in some manner the transport of glucose and other stereospecific sugars into certain cells has been amply demonstrated. I would agree with Professor Cori's remark that this does not mean that we know the mechanism of action. We are perhaps nearer to it but we still do not know the mechanism of action of insulin. The fact that many substances and conditions other than insulin may be shown also to have an effect on the transport system we should like to explain in the following manner. The transport system is probably present in all cells metabolizing sugar yet some cells do not show sensitivity to the action of insulin. We believe therefore that insulin probably does not act directly on the transport system itself. However certain cell systems such as muscle, the connective tissues and the specialized connective tissues like fat cells may have this transport system shielded under most conditions in the absence of insulin. This explains the fact that in a fasting animal where the total output of sugar



by the liver is small where about two-thirds of that output is utilized by brain that the animal does not become acutely hypoglycaemic. Most of the metabolic mass which can utilize sugar like muscle and fat tissue is closed off so to speak or its transport gates are firmly locked. However these locks can be removed hormonally by insulin under artificial conditions by certain agents like dinitrophenol or anaerobiosis and by physiological conditions like muscular work. This therefore means that we have to look for something which ordinarily inhibits transport in the insulin-sensitive tissues and that insulin removes this inhibition.

Perhaps the findings of Dr Park that under the influence of anterior pituitary hormone transport is inhibited may lead to the elucidation of the nature of the cover for the transport system itself.

Now as to the differences between glucose at high concentration which should give the same results as does insulin but under certain conditions does not. I believe in the first place that one can cull from the literature many individual experiments in which perhaps all of the actions of insulin have been imitated by high sugar administration perhaps not from the same laboratory not at the same tissue but as a general picture. The intracellular enzymatic systems in the tissues are controlled by factors other than hormones. Let us for example take  $\text{CO}_2$  production. The rate of the Krebs cycle for example is controlled by factors other than insulin and may in a diaphragm taken from a normal animal be already at a height which cannot be stimulated by additional glucose entry. In other words the conditions are set in the cell. If one takes the diaphragm from an alloxanized animal the amount of isotopic  $\text{CO}_2$  production from glucose is certainly less than in the normal animal. At the present moment in spite of the very interesting experiments of Professor Chain it would be too premature to give up the notion that the most important, if not the sole action of insulin on peripheral tissues including muscle heart and fat tissue is to remove an inhibition to the normal rate of transport of sugars into the cell interior and that the state of the interior then determines the fate of the sugar.

YOUNG: Would you like to comment briefly Professor Chain?

CHAIN: Let us first come to the rather essential point whether insulin does or does not increase  $\text{CO}_2$  production. We are certain that it does not in the rat diaphragm under conditions in which there is a marked insulin effect on the polymerization of glucose and the same applies to the brown adipose tissue. I should perhaps mention that as Dr Levine has pointed out some authors have reported a stimulating effect of insulin on  $\text{CO}_2$  production in the isolated rat diaphragm and for this reason we have carried out a very large number of experiments to see whether or not we could confirm such effect. Though in a few experiments we noticed an apparent effect this was not found to be statistically significant.

but simply due to biological variations. It is axiomatic, but not always remembered by all investigators, that no cogent conclusions can be drawn in this field of research unless a sufficiently large number of experiments to allow statistical analysis is carried out. It is also certain from our experiments that insulin does not produce an increase in lactic-acid formation from glucose while increasing the external glucose concentration in the incubation medium markedly stimulates both  $\text{CO}_2$  production and lactic acid production.

Finally it is not possible to increase the formation of fat by increasing the external glucose concentration in the white adipose tissue above a threshold value of about 0.3 per cent. In the spectacular way insulin does in fact it would be difficult to understand thermodynamically how a series of reactions as endergonic as fat synthesis could be stimulated by changes of concentration of a substrate like glucose within physiological range which is removed by many reaction stages from the end product.

In my own view these facts show irrefutably that the mode of action of insulin cannot be explained by a theory like the permeability theory which postulates that insulin acts by increasing the intracellular concentration of glucose.

We are far from offering a detailed alternative explanation of the mode of action of insulin. All we can say at present is that insulin enables the tissue cells on which it acts to carry out a larger amount of energy requiring synthetic reactions (polysaccharides, fat and protein synthesis) than it can accomplish in its absence. Several mechanisms by which this could be done can be visualized and as has been already mentioned this is one of the main problems which form the object of our present research.

I should like some member of this audience of distinguished specialists in the field of intermediate carbohydrate metabolism to offer some explanation of the results of our anaerobic experiments. There is no question that without oxygen there is no glucose metabolism. Why is this so? Some factor which no one seems to have discussed yet is evidently missing which brings oxygen into the scheme of glucose metabolism and glycogen synthesis. We do not know the nature of this factor but it is certainly not ATP and I feel that without knowledge of this factor it will be difficult to understand the mode of action of insulin.

KRAHL: Leloir has shown that glycogen can be formed by liver from uridine diphosphoglucose. The possibility has occurred to me that there may be an insulin stimulation of a pathway from glucose to glycogen via uridine diphosphoglucose or other analogous nucleotides. I should like to ask Professor Charn whether with his techniques he could identify such intermediates in muscle and measure their concentration and radioactivity in relation to insulin effects.

CHARN: It is interesting that you should have mentioned this. This is

of course a field in which we are actively engaged at the present. We also believe that something of that sort is involved in the transport of glucose and glucose utilization

We have got some radioactive nucleotides out of the rat diaphragm, and we have got them particularly out of liver but we are just in the process of identifying them and I would not like to say too much about them except that one of the liver nucleotides which we have got shows a high specific activity indicating a considerable turnover. But this is a problem under active investigation

CORI I should like to mention that these uridine nucleotides are formed by way of glucose 1 phosphate. At the present time there is no other mechanism known for their formation.

Secondly the Leloir enzyme is present in low concentration both in liver and muscle about one-hundredth the concentration of phosphorylase. Now while the enzyme exists it does not necessarily follow that it functions. If it could function in the direction of synthesis phosphorylase could have its main function as a glycogenolytic enzyme. This is a possible mechanism.

CHAIN May I ask Professor Cori it is true that at present there is no pathway known apart from the classical one but does that necessarily mean that no other pathway exists?

CORI I didn't say that no other pathway exists. I merely said that in order to form uridine diphosphoglucose you have to go through glucose 1 phosphate. Now the glucose 1-phosphate could be utilized directly by the phosphorylase or it could go by another enzymatic step to uridine diphosphoglucose and then be used by the Leloir enzyme but this enzyme is not very active. The question is whether the activity of this enzyme is of sufficient magnitude to explain the rate of glycogen formation in liver and muscle.

LEVINE Professor Chain contrasts the seeming complexity of the transport theory with his simple concept. Insulin somehow raises the energetic level. Such a statement is far from simple. No theory proposed for the action of insulin has yet reached the molecular level. I do believe however that there is a certain amount of evidence for the facilitation of penetration especially when one considers that such materials as galactose, xylose, arabinose and others which are not phosphorylated to any extent in peripheral tissues are aided in their penetration into various cells by insulin.

BESSMAN Professor Chain's mention of our results prompts me to present them together with our reasons for performing such experiments.

We tested the possibility that insulin might act on carbohydrate metabolism in tissue essentially deprived of a membrane by cutting rat diaphragm into pieces so small (1-3 millimetres square) that d-xylose

could penetrate them to the same extent without insulin as it could penetrate the intact diaphragm with insulin. The addition of insulin to such pieces does not enhance the penetration of xylose significantly. When such pieces are included in a medium containing both glucose and xylose there is the same penetration of xylose with or without insulin but glycogen synthesis occurs only in the presence of insulin. Since there is apparently good permeability in this preparation without insulin and yet there is no glycogen synthesis when such penetration has occurred we interpret these data to mean that the role of insulin in stimulating glycogen synthesis is not primarily the effect of insulin upon cell permeability. Insulin obviously acts on the cell to improve its permeability but this is the result of a direct effect upon carbohydrate utilization as our experiments with damaged cells demonstrate.

This brings me to the reason for such an experiment. The preoccupation with membrane transport as the site of action of insulin has led to a general neglect of what appears to be a more specific site of action of insulin namely the energy generating system. Professor Chain has pointed out a number of metabolic peculiarities resulting from insulin action which cannot be explained on the basis of a direct effect of insulin on transport of substrate across cell membranes. I should like to point out certain other highly improbable assumptions which must be made to support a transport theory of the action of insulin. The effect of insulin on transport now embraces a large and varied group of compounds including sugars, natural and unnatural amino acids of every variety and potassium ions. The likelihood that all of these substances are moved into the cell by the same mechanism is very small indeed. Secondly the effect of toxic substances such as cyanide and dinitrophenol on facilitating membrane permeability suggests that energy metabolism is the question not specific transport mechanisms. The effect of exercise on stimulating carbohydrate uptake without insulin also calls for supplementary assumptions and finally the relation of different tissues all of which utilize glucose to the syndrome of insulin deficiency is not explained by a permeability hypothesis. I mean by this that brain which uses glucose almost exclusively does not require insulin at all that diabetic muscle has a normal ability to utilize all substrates except glucose and that diabetic liver apparently can utilize no substrate normally.

For these reasons among others we have attempted to develop a unified hypothesis for the site of action of insulin. We believe that insulin connects mechanically the enzyme hexokinase to the mitochondrion. This function of insulin as a biochemical organizer would produce all of the physiological effects which have been ascribed to insulin.

It is well known that mitochondria oxidize inefficiently in the absence of an acceptor of ATP which can return ADP to the site of oxidative

phosphorylation The attachment of hexokinase to the mitochondria provides such an acceptor Brain mitochondria in contradistinction to the mitochondria of the rest of the body have hexokinase attached firmly This obviates the need for the proposed function of insulin in brain

Muscle has an excellent acceptor in the form of the creatine kinase-creatine system and requires no additional acceptor for normal oxidation In fact exercise which stimulates turnover in the creatine kinase system stimulates diabetic muscle to take up glucose

The membrane transport mechanisms and I emphasize the plural, all require energy which must be furnished by an efficiently functioning mitochondrial system From such a system comes the energy for protein synthesis acetylation phosphorylation of thiamine and the many other anabolic functions which are deficient in the diabetic state

In order to accept the transport theory of insulin action we are obliged to say that the deranged metabolism of the diabetic liver is completely secondary to the minor change in muscle metabolism caused by insulin lack From the acceptor-insulin hypothesis however we can see that the abnormal metabolism of the liver results from the lack of an adequate acceptor system equivalent to muscle The liver requires the hexokinase system to be attached to the mitochondria for there is very little creatine kinase activity in liver therefore the liver is more dependent on insulin for energy metabolism than muscle

Thus we have an opportunity to unify the physiological picture of the requirement of different organs for insulin with a single site and mechanism of action of insulin the connection of hexokinase to mitochondria to provide an acceptor reaction for energy generation (*A Contribution to the Mechanism of Diabetes Mellitus* p 133 in *Fat Metabolism* edited by Victor A Najar The Johns Hopkins Press Baltimore 1954)

PART IV  
INSULIN AND THE LIVER  
Chairman PROFESSOR C F CORI



# INSULIN AND GLYCOGEN SYNTHESIS IN THE LIVER

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The problem of the action of insulin on glycogen synthesis in the liver has been dealt with before in publications from Professor Bouckaert's laboratory and from our own (Berthet *et al.*<sup>1</sup> Bouckaert and de Duve<sup>2</sup> de Duve<sup>3</sup> and Hers and Bouckaert<sup>4</sup>) but remains a chronic one. The most recent attempt to solve it has been made by Levin and Weinhouse.<sup>5</sup> These authors have made a careful time-course study over periods ranging between 5 minutes and 4 hours of the incorporation of radioactivity in liver and muscle glycogen and in blood CO<sub>2</sub> on rats injected with a trace dose of uniformly labelled <sup>14</sup>C glucose with or without insulin (20 U/kg). Three groups of animals were used: rats fasted 20 to 48 hours with a very low initial level of hepatic glycogen; similar animals receiving 0.9 g of unlabelled glucose by stomach tube at the beginning of the experiment; and post absorptive rats having a liver glycogen content of approximately 2 per cent. The administration of insulin inhibited the net synthesis of liver glycogen in the first two groups and caused glycogenolysis in the third; it prevented almost completely the incorporation of labelled glucose into hepatic glycogen in all three groups while stimulating the appearance of <sup>14</sup>C in muscle glycogen and blood CO<sub>2</sub>. Hypoglycaemia occurred in animals even in those receiving glucose. The authors sum up results as follows: Under the conditions employed here none of the glucose which disappeared quickly from the blood; the influence of insulin entered the liver glycogen despite the marked effect in muscle.

There can of course be no quarrel with this statement but one may question whether the conditions employed are appropriate for the study of the physiological effects of

The authors use glucagon free insulin and therefore avoid one of the dangers which we have denounced before: even they furnish no evidence that the insulin they have is fact entirely devoid of glucagon. In our experience some



purported to be glucagon-free and causing no appreciable initial hyperglycaemia when injected intravenously into the intact animal, still contain measurable amounts of glucagon, as assayed by the more sensitive liver-slice or phosphorylase methods. Such amounts could inhibit glycogen synthesis during the early part of treatment but it is doubtful that their effect could extend over the full experimental period of 2 to 4 hours.

Much more damaging to the validity of the experiments is the fact that practically all the animals were hypoglycaemic at the time of sacrifice. The strong dependence of the glucose-glycogen balance of the liver on the blood-sugar level has been repeatedly emphasized. In the present case the authors rule out a disturbing influence of hypoglycaemia on the grounds that insulin failed to stimulate the incorporation of radioactive glucose into liver glycogen even in the post-absorptive animals with high glycogen stores.

However this reasoning would be correct only if the synthesis and breakdown of glycogen occurred by two distinct processes and if there were a true pool of glycogen in which incorporated radioactive units immediately became strongly diluted. The first assumption is obviously invalid if phosphorylase is involved in both the formation and rupture of the 1,4  $\alpha$ -glucosidic linkages in glycogen. Even assuming as has lately been suggested that phosphorylase acts only in glycogenolysis and that another enzyme system is responsible for the synthesis of glycogen it is highly probable that the newly incorporated glucosyl units will be added to the outer tiers of the glycogen molecule therefore at a site where they will be most vulnerable to the action of phosphorylase. Any acceleration of glycogenolysis will therefore effectively counteract an increased formation of radioactive glycogen from  $^{14}\text{C}$  glucose unless the rearrangement of the glycogen molecules by branching processes is an extremely rapid phenomenon. That such is not the case has been demonstrated by Stetten and Stetten<sup>9</sup> and confirmed in our laboratory. An example of this is shown in Fig. 1: labelled glucose units incorporated under the influence of insulin are seen to disappear very rapidly from the glycogen as conditions become less favourable.

In short whether glycogen synthesis is measured chemically or by the incorporation of radioactive glucose the same precautions must be strictly adhered to in experiments designed to explore the role played by insulin in the physiological conversion of glucose to liver glycogen.

(a) The insulin used must be entirely free of glucagon

(b) The animals must be kept at a normal or slightly elevated blood-sugar level by an exogenous supply of glucose during the whole experimental period since this is the state in which the physiological secretion of insulin takes place

As has been reported in previous publications (Berthet *et al.*<sup>1</sup> de Duve<sup>2</sup>) a significant stimulation of hepatic glycogen synthesis by administered insulin has been repeatedly evidenced under the above conditions both by the chemical and by the isotopic methods. It has further been shown that the extra glycogen synthesized under the influence of insulin is derived directly from circulating glucose and not from metabolites such as lactate or pyruvate formed in increased amounts by the peripheral tissues. Finally an *in vitro* effect of insulin on hepatic glycogen synthesis has been demonstrated on isolated liver slices.

An almost perfect counter-demonstration of these results is provided by the recently published data of Spito *et al.*<sup>3</sup> who have shown that the hepatic glycogen stores as well as the ability of the isolated tissue to convert glucose to glycogen decrease rapidly in insulin-treated alloxan-diabetic rats as a result of insulin deprivation and are then restored by the administration of insulin.

In view of these results and of numerous previous ones we see no reason at the present time to alter our view that insulin plays an essential and direct role in the physiological deposition of alimentary glucose as liver glycogen. The fact that an action of insulin can be demonstrated on muscle or adipose tissue under conditions where no hepatic effects of the hormone are seen in our opinion is irrelevant to the main discussion since the liver occupies a unique metabolic position shared only to some extent with the kidneys as a glucose-forming organ. Neither do we see an objection in the well-documented finding (see for instance Winternitz *et al.*<sup>10</sup>) that the formation of liver glycogen can be influenced independently of insulin by adrenal steroids. The effect of the latter obtains with a variety of precursors and obviously differs in its mechanism from that of insulin which appears to be fairly specific.

Recent investigations carried out by Dr P. Jacques in our laboratory have provided some additional information on the action of insulin on isolated liver slices reported by Berthet *et al.*<sup>1</sup> The results obtained on glycogen formation in these new experim

are summarized in Table I. It will be noticed that a significant effect of insulin is obtained with glucose but not with fructose as precursor and that this effect is maximal between the 60th and 90th minutes of incubation.

A careful statistical examination of the results after 30 minutes indicates that the value of 10.5 per cent is not truly representative of the insulin effect but arises from the summation of a large number of experiments in which the hormonal effect was negligible with a limited number of cases where the insulin stimulation ranged between 60 and 150 per cent. The proportion of negative results appeared

TABLE I

EFFECT OF ADDED INSULIN ON SYNTHESIS OF GLYCOGEN  $^{14}\text{C}$  BY ISOLATED RABBIT LIVER SLICES  
Experimental conditions of Berthet *et al.*<sup>1</sup> unpublished results of P. Jacques

Substrate	Incubation time min	Number of comparisons	Percentage stimulation by insulin	t	P
Glucose- $^{14}\text{C}$	30	91	$+10.5 \pm 3.1$	3.40	0.001
	60	21	$+23.7 \pm 5.4$	4.40	<0.001
	90	14	$+34.9 \pm 9.3$	6.8	0.0
	120	7	$+21.0 \pm 11.6$	1.81	—
Glucose- $^3\text{C}$ + fructose	30	19	$+10.6 \pm 4.1$	2.60	<0.05
Fructose- $^{14}\text{C}$	30	30	$+2.7 \pm 4.3$	0.63	—
Fructose- $^3\text{C}$ + glucose	30	23	$+2.4 \pm 6.0$	0.40	—

to be smaller in the longer term experiments but would presumably increase again if the incubation were extended over more than 2 hours (see Fig. 1).

Dr Jacques has also studied the conversion of glucose and fructose carbon to  $\text{CO}_2$  in a limited number of experiments. His results are shown in Table II. As is seen there are indications of a stimulation by insulin of the oxidation of the first glucose carbon and of fructose. In all these experiments a significant positive correlation was observed between the effects exerted by insulin on the formation of glycogen and on the production of  $\text{CO}_2$  by the same liver slice. This correlation was particularly striking ( $P < 0.001$ ) when mixtures of the two hexoses with either one or the other labelled were used as substrates.

The first conclusion to be drawn from these new results is that the observed effects of insulin are often of borderline magnitude and can easily escape detection unless carefully paired slices are used and the incubation is stopped at an appropriate time. This however does not necessarily mean that they are artifacts devoid of physiological significance. The results of Fig. 1 which were all obtained on slices from the same liver should suffice to render such a hypothesis very unlikely. Besides illustrating clearly the importance of time and the preferential effects of insulin on the conversion of

TABLE II

EFFECT OF ADDED INSULIN ON FORMATION OF  $^{14}\text{CO}_2$  BY ISOLATED RABBIT LIVER SLICES  
Unpublished results (P. Jacq. et al.)

Substrate	Incubation time (min)	Number of experiments	Percentage increase in $^{14}\text{CO}_2$ formation by insulin	t	P
Glucose- $^{14}\text{C}$	30	20	$+ 33 \pm 5.5$	0.6	—
Glucose- $^{14}\text{C}$	30 60	6 6	$- 33 \pm 6.4$ $+ 23 \pm 9$	0.43 2.36	— 0.05
Glucose- $^{14}\text{C}$ + fructose	30	20	$+ 39 \pm 4.6$	0.85	—
Glucose- $^{14}\text{C}$ + fructose	30	6	$+ 95 \pm 4.5$	3.11	<0.10
Fructose- $^{14}\text{C}$	30	11	$+ 23.7 \pm 10.7$	3.20	0.05
Fructose- $^{14}\text{C}$ + glucose	30	8	$+ 34 \pm 23$	0.15	—

glucose to glycogen these results indicate further that a mild treatment of the hormone with alkali which suppresses its hypoglycaemic action also destroys its ability to influence glycogen synthesis in isolated liver slices.

A more reasonable conclusion suggested by the experimental facts is that two factors—one present in fresh slices and decreasing in efficiency as the incubation proceeds, the other appearing during incubation—combine to prevent the effect of insulin from becoming evident *in vitro*. Only when the combined influences of the two factors are minimal, i.e. on an average between the 60th and 90th

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Fructose- $^{14}\text{C}$ + glucose	30	3	$+2.4 \pm 6.0$	0.40	—

to be smaller in the longer term experiments but would presumably increase again if the incubation were extended over more than 2 hours (see Fig. 1).

Dr Jacques has also studied the conversion of glucose and fructose carbon to  $\text{CO}_2$  in a limited number of experiments. His results are shown in Table II. As is seen there are indications of a stimulation by insulin of the oxidation of the first glucose carbon and of fructose. In all these experiments a significant positive correlation was observed between the effects exerted by insulin on the formation of glycogen and on the production of  $\text{CO}_2$  by the same liver slice. This correlation was particularly striking ( $P < 0.001$ ) when mixtures of the two hexoses with either one or the other labelled were used as substrates.

account for the increased synthesis of glycogen and oxidation of the first carbon of the glucose molecule as well as for the general correlation between glycogen formation and  $\text{CO}_2$  production

As shown in experiments performed in the absence of insulin an increased utilization of unlabelled glucose by the liver slices would tend to depress the formation of radioactive glycogen and to increase the production of  $^{14}\text{CO}_2$  from radioactive fructose. Thus the observed stimulation of the latter process by insulin could be explained simply as a consequence of the effect on glucose utilization but if this were the case one should expect a negative correlation between the effects of insulin on glycogen and  $\text{CO}_2$  formation from fructose. As mentioned earlier the correlation is positive and it appears therefore that some direct effect of insulin on the utilization of fructose also takes place. Possibly as has been observed on the isolated rat diaphragm (Hers<sup>10</sup>, Mackler and Guest<sup>7</sup>) this effect may be restricted to a pathway of fructose uptake mediated by hexokinase.

In conclusion the results described in this paper are in line with those of other authors who have described effects of insulin on fatty acid and protein synthesis in isolated liver slices. They provide additional evidence that insulin stimulates directly the uptake of glucose by the liver at least under some conditions.

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minutes of incubation under the conditions of our experiments are the influences of insulin clearly observable. Quite possibly this may never happen under other experimental conditions.

We do not know what these two factors may be. As far as the first one is concerned it is possible since fed animals were used that the tissue used is practically saturated with insulin at the start and

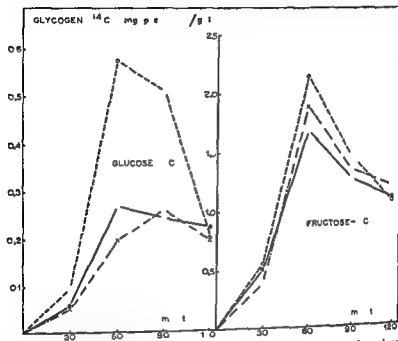


FIG. 1. Incorporation of labelled hexoses into the glycogen of isolated rabbit liver slices

○—○ Controls  
 ○—○ Incubated with insulin (1 U/ml)  
 ×—× Incubated with insulin previously inactivated by a 3 hour incubation at 37 in 0.1 N KOH

that the hormone present in the control slices must first become inactivated for a differential effect to become evident. A deterioration of the insulin-responsive enzyme systems with time could account for the second effect. However, there are many alternative explanations and nothing definite can be said at the present time.

As to the nature of the insulin effect it is best explained in terms of a stimulation of the primary phosphorylation of glucose or of a transport phenomenon preceding this process since this would

# INFLUENCE OF INSULIN ON THE GLUCOSE METABOLISM OF THE LIVER

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In previous studies of the glucose turnover in the body the single injection of  $^{14}\text{C}$ -labelled glucose has been applied<sup>1</sup> as the rate of glucose release and uptake can be calculated from the exponential decrease of the plasma glucose specific activity. However it is conditional that the release of glucose into and the uptake of glucose from the extracellular pool is maintained in a steady state. Thus it will not be possible to obtain reliable information about the influence of insulin upon the glucose metabolism from such data as the steady state is outbalanced as soon as the hormone is administered and the plasma glucose concentration does not any longer represent the concentration in the various compartments of the pool.

In order to be able to measure the rate of glucose release from the liver even during actual changes in the size of the glucose pool a method has been worked out in which the pool size does not enter into the calculations. As the specific activity of plasma glucose in portal and arterial blood is identical in the fasting state the amount of unlabelled glucose released by the liver can be calculated by setting the ratio of the specific activity of arterial plasma glucose to hepatic venous glucose equal to the ratio of the amount of glucose passing through the liver to the total amount leaving the liver.

This method is applied to a study of the influence of intraportally and intravenously administered insulin upon the release of glucose from the liver.

## MATERIALS AND METHODS

The experiments were performed on normal cyclopropane anaesthetized dogs which received their last meal 18 hours before. A hepatic vein was catheterized under fluoroscopic control and infusion of Bromsulphalein (BSP) was applied in order to estimate the hepatic plasma flow.<sup>2</sup> After an equilibration period following the





If  $\gamma A$  and  $\gamma H$  represent the specific activity of glucose in arterial and hepatic venous blood the ratio  $\gamma A/\gamma H$  will be equal to the ratio between the amount of glucose leaving the liver ( $Q$ ) and the amount passing through the liver ( $D$ ) hence

$$\frac{\gamma A}{\gamma H} = \frac{Q}{D}$$

While  $D$  cannot be ascertained the value of  $Q$  is calculated from the estimated hepatic blood flow and the hepatic-venous glucose concentration and further

$$Q = D + a$$

where  $a$  is the amount of unlabelled glucose released from the liver this amount can be derived as

$$a = Q \left( 1 - \frac{\gamma H}{\gamma A} \right) \quad (1)$$

This equation will be suitable for liver output calculations even in periods where a steady state with respect to glucose metabolism is not maintained as variations in the glucose pool do not interfere

The glucose output calculated according to this procedure is shown in Fig. 3 and Table I together with the net amount of plasma glucose leaving the splanchnic organs calculated from the difference in glucose concentration between a hepatic vein and the femoral artery multiplied by the estimated hepatic plasma flow

### RESULTS

In Fig. 2 the data presented was obtained in a normal dog which was infused intraportally and 14 days later intravenously with 0.08 i.u./kg/hr of glucagon free insulin. The drop in blood sugar was significantly smaller when the hormone was administered via the portal vein. This observation confirms the findings of Weisberg *et al.*<sup>8</sup> who also showed that a content of glucagon in the insulin preparation could not account for the observed difference. They explained this phenomenon by assuming that the liver or more specifically the insulinase in the liver retains or inactivates a greater part of the insulin which is administered via the portal vein.

The glucose output of the liver before and during an intraportal infusion of 0.08 i.u. of insulin/kg/hr is shown in Fig. 3. Although this dose of insulin does not have any apparent effect upon the arterial blood-sugar concentration it could be anticipated that if

intravenous injection of a dose of randomly labelled  $^{14}\text{C}$  glucose (200  $\mu\text{C}$ ) blood samples were collected simultaneously from the hepatic vein and the femoral artery every 15 minutes for 1-2 hours which served as a control period. Infusions of glucagon free insulin were then started at rates varying from 0.02-0.12 u/kg/hr either into a peripheral vein or the portal vein into which a catheter had been inserted via a small jejunal vein at least 4 days prior to the experiments.

Plasma glucose was determined according to Nelson<sup>15</sup> and Somogyi<sup>16</sup> and BSP concentration according to Gaebler<sup>7</sup> and

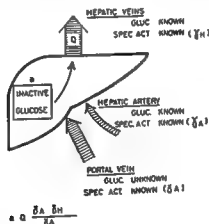


FIG. 1 The principle of the isotope dilution method worked out for calculating the release of unlabelled glucose (a) from the liver ( $Q$  = total amount of glucose leaving the liver cf text)

Munkner<sup>11</sup> Determinations of the glucose specific activity were performed as previously described<sup>18</sup>

The principles of the calculation of the amount of unlabelled glucose released from the liver are shown schematically in Fig. 1. The specific activity of the glucose in portal and in arterial blood is identical if no dilution with unlabelled glucose absorbed from the intestines occurs. This is confirmed experimentally in normal dogs starved for 18 hours although the concentration of glucose may differ in blood from these two vessels. The arterial specific activity can thus be regarded as representative for all blood glucose entering the liver.

insulin affects the glucose uptake of the liver cells this dose given intraportally might be followed by an alteration in the net amount of glucose leaving the liver and also in its release of unlabelled glucose. This however was not the case as the values remained approximately constant for the whole infusion period although the hepatic cells presumably were exposed to concentrations of insulin which would stimulate the glucose uptake of the peripheral tissues.

Similar results were obtained with intraportal infusions of 0.04, 0.08 and 0.12 i.u./kg/hr. In Table I the arterial and hepatic-venous plasma glucose and its specific activity are listed together with the calculated plasma output from the liver of unlabelled glucose before and during an infusion of 0.12 i.u./kg/hr intraportally. The decrease in the arterial blood sugar only indicates an increased uptake of glucose in the peripheral tissues as the output from the liver remained almost constant.

TABLE I

EFFECT OF PORTAL INFUSION OF INSULIN (0.12 I.U./KG/HR.) UPON THE ARTERIAL AND HEPATIC-VEINOUS PLASMA GLUCOSE CONCENTRATIONS AND THE OUTPUT OF UNLABELLED GLUCOSE FROM THE LIVER OF A NORMAL DOG

M. from start of inf. in	Femoral artery		Hepatic vein		Plasma glucose out- put <sup>†</sup> mg/min
	mg/gl 100 ml pl	c.p.m./mg glucose $\times 10^{-3}$	mg glucose/ 100 ml plasma	c.p.m./mg glucose $\times 10^{-3}$	
-75	121	323	118	276	39
-60	115	81	139	232	41
-45	116	238	137	197	37
-30	117	197	138	166	34
-15	111	169	141	143	31
0	104	147	141	123	31
15	96	124	134	100	30
30	84	105	118	80	43
45	71	84	100	69	29
60	67	66	92	55	3
75	60	57	81	45	22

The hepatic plasma flow averaged 150 ml./min.

† Calculated from the equation.<sup>1</sup>

## DISCUSSION

The results obtained after intraportal infusion of relatively small doses of glucagon free insulin into cyclopropane anaesthetized normal dogs indicate that the glucose output from the liver is not, even partly inhibited by the influence of exogenous insulin. The

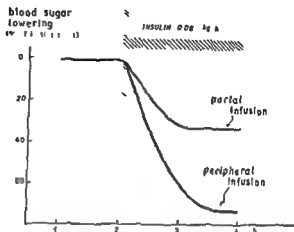


FIG. 2 Comparison of the hypoglycaemic effect of intraportally and intravenously infused insulin into the same normal anesthetized dog

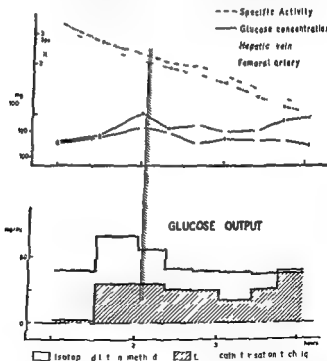


FIG. 3 Effect of intraportally infused insulin ( $0.06 \text{ kg/hr}$ ) into a normal dog upon the output of unlabelled glucose from the liver and the net amount of glucose leaving the splanchnic organs. In the upper part of the figure are shown the arterial and hepatic venous plasma glucose concentration and specific activity.

carried out in animals in the post-absorptive state in which the release of glucose from the liver has been the dominating process responsible for the upkeep of a constant blood sugar. In addition insulin has nearly always been administered intravenously or subcutaneously and thus been offered the chance of being bound to or affecting the peripheral tissues far more than the liver and thereby shifting the whole glucose transport into a more pronounced one-sided process in the direction from the liver to these tissues than before.

Future experiments probably have to be designed so the influence of insulin can be investigated upon the liver of intact animals which are not only receiving physiological amounts of insulin via the portal venous blood but also are maintained in a state where the glucose metabolism has what might be defined as an inward hepatic trend.

#### SUMMARY

1. The inadequacy of the isotope dilution method after a single injection of  $^{14}\text{C}$  glucose for measuring smaller variations in the glucose release from the liver during changes in the size of the glucose pool prompted the development of a method which permitted the calculation of the amount of unlabelled glucose released by the liver at any time during changes in the plasma glucose concentration. This was done by relating the specific activity of hepatic-venous and arterial blood sugar to the directly measured total amount of glucose leaving the splanchnic system.

2. This method was applied in studying the effect of intraportal and intravenous insulin infusion of from 0.0–0.1 u/kg/hr into normal dogs. As previously observed the hypoglycaemic effect obtained after intraportal infusion is 40–50 per cent smaller than after intravenous infusion of an identical dose but no decrease in the hepatic glucose output was found.

3. It is suggested that future experiments should be designed so that the effect of intraportally administered insulin is studied in a liver which is already taking up glucose.

The authors wish to express their appreciation to Dr K. Hallas-Møller for valuable suggestions and criticism and their indebtedness to Professor Aa. Thordahl-Christensen DVM at the Royal Veterinary and Agricultural College Copenhagen for placing the  $\lambda$  ray equipment of his department at their disposal.

investigation which combines the phys<sup>1</sup> introducing insulin into the blood with mea<sup>1</sup> of glucose released from the liver alone, should sidered in relation to previous experimental insulin effect upon the liver was studied in o<sup>1</sup>

Many investigators have not been able to effect upon the glucose uptake or release of the<sup>1</sup> fusion experiments<sup>9 12 13</sup> in liver slices<sup>15</sup> or intact the liver was suddenly excluded from the blood

On the other hand a decrease in the glucose<sup>1</sup> by liver catheterization of patients<sup>1</sup> or with an technique using either a single injection<sup>4</sup> or a co<sup>14</sup>C glucose<sup>19</sup> although the authors of the latter of the decrease in the blood sugar is predominantly peripheral glucose uptake. However, our studies with did not show any evidence of an inhibition of the release of 0.15 i.u. of insulin/kg. were injected intravenously. Berthel *et al.*<sup>2</sup> found that insulin *in vitro* stimulates the deposition of radioactive glucose into the glycogen of<sup>1</sup>

None of these results present the necessary conclusive nor does the work which is characterized by the co ministration of glucose to obviate the fall in blood insulinized preparation. In such dogs de Duve *et al.*<sup>5</sup> for the necessary amount of glucose was reduced to about b, hepatectomy while Lundsgaard<sup>10</sup> could not confirm this o tion in cats. Lang *et al.*<sup>8</sup> found in dogs an almost similar<sup>1</sup> as de Duve although they interpreted this to be due to a factor which stimulated the peripheral utilization of gl did not believe that the utilization of the liver was causing difference.

The interesting data of Cahill *et al.* who demonstrated a free meability of the liver cell membrane with respect to glucose cluded any effect of insulin upon the transport of glucose across hepatic cell membrane but suggested that the rate at least determined by the activity of glucokinase, glucose<sup>1</sup> the size of the glucose-6-phosphate pool and the level of circulating glucose. If the hormone does have an effect on these factors thereby on the further transport of glucose into the metabolic pathways then it should be studied under such circumstances that the liver cells already take up glucose. Previous work has mainly<sup>1</sup>

## DISCUSSION

MAHLER. We have attempted, in as direct a physiological manner as possible, to solve the problem of the immediate effect of insulin on glucose production by the liver. Plastic catheters were placed at operation in the

TABLE I  
MEANS OF RESULTS IN NINE NORMAL DOGS

	Control period	Minutes after injection of 1 ml			
		5	10	15	30
Hepatic plasma flow ml/min/kg	30.2	32.4	37.4	33.4	34.0
Difference between hepatic and portal plasma glucose mg/100 ml	9.9	13.7	13.7	10.7	18.4
Hepatic glucose output mg/min/kg	3.1	4.3	5.1	3.5	6.3

portal and hepatic veins of normal dogs a few days before any further experiments were undertaken. In this way it was possible to obtain blood entering and leaving the liver at any instant without the metabolic disturbances of acute surgery and anaesthesia.

TABLE II  
EFFECT OF INSULIN ON RECYCLING OF  $^{14}\text{C}$

Recycling is expressed as the percentage of  $^{14}\text{C}$  appearing in carbon atom 1 of plasma glucose after the injection of a tracer dose of glucose labelled at carbon atom III.

Time from injection of $^{14}\text{C}$ glucose	Recycling of about 1 carbon atom		
	Percentage of plasma glucose specific activity		
Minutes	Dog C-34	Dog C-35	Dog C-41
15	3.8	—	0
25	6.2	5.0	—
55	7.3	4.4	5.2
Injection of insulin			
40	17	7.3	16
45	18.8	11.8	13
50	19	16.8	—
65	35	—	40

Hepatic glucose production: the amount of glucose added by the liver to the blood flowing into it, could then be calculated from the hepatic blood flow and the difference in glucose concentration in hepatic and portal vein plasma.



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Recycling is expressed as the percentage of  $^{14}C$  appearing in carbon atom 1 of plasma glucose after the injection of a tracer dose of glucose labelled at carbon atom 6.

Time of analysis of $^{14}C$ glucose	Recycling of $^{14}C$ at carbons		
	Percentage of plasma glucose specific activity		
	Dose C-38	Dose C-39	Dose C-43
Minutes			
15	3.8	—	0
35	6.2	5.0	—
35	7.2	4.4	5.2
Injection of insulin			
40	17	7.2	11
45	18.8	11.8	12
50	19	16.8	—
65	35	—	40

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TABLE II  
EFFECT OF INSULIN ON RECYCLING OF  $^{14}\text{C}$

Recycling is expressed as the percentage of  $^{14}\text{C}$  appearing in carbon atom 1 of plasma glucose after the injection of a 1 dose of glucose labelled at carbon atom 6

Time min after injection of $^{14}\text{C}$	Recovery of active carbon atoms		
	Percentage of plasma glucose		
Mean	D 2 C 38	D 2 C-39	D 2 C 43
15	3.8	—	0
25	6.2	5.0	—
35	7.2	4.4	5.2
Interval			
40	17	7.2	16
45	18.8	11.8	12
50	19	16.8	—
65	35	—	40

Hepatic glucose production: the amount of glucose added by the liver to the blood flowing into it could then be calculated from the hepatic blood flow and the difference in glucose concentration in hepatic and portal vein plasma.

Before the injection of insulin the difference in glucose concentration between the hepatic and portal vein was 9.9 mg per 100 ml and hepatic glucose production 3.1 mg per minute per kg body weight. Immediately after the injection of 0.2 units per kg of glucagon free insulin glucose concentration dropped in both the hepatic and portal veins. However the concentration gradient between them did not change significantly and there was no decrease in hepatic glucose production. In fact the

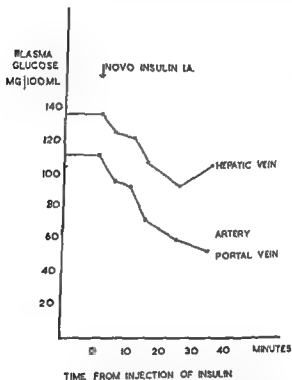


FIG. 1 The effect of a single injection of glucagon free insulin on glucose concentration. (Dog C-32)

only significant change was an increase in glucose production in response to the hypoglycaemia when the portal glucose concentration had dropped to 60 mg per 100 ml or less.

The fall in blood-sugar concentration in normal dogs in response to insulin is therefore not due to any effect of insulin on glucose production by the liver but results from an increase in glucose utilization by insulin sensitive tissues.

From the reversal after insulin of the glucose concentration difference between the artery and portal vein it is evident that the tissues of the splanchnic area which include the mesenteric fat take part in this response. Consequently calculations of hepatic glucose output based on concentration differences between hepatic vein and artery give misleading results (Bearn *et al* *Clin Sci* 1952 **11** 151)

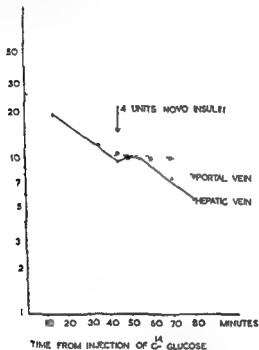


FIG 2 Change in specific activity of plasma glucose before and after administration of insulin (D G C-32)

After the injection of a tracer dose of  $C^{14}$ -labelled glucose its specific activity in the plasma decays at an exponential rate.

The injection of insulin is immediately followed by a change in the rate of decay for a variable period of time. In some experiments there appears to be a plateauing of the curve and this has been interpreted by some (Dunn *et al* *J Biol Chem* 1957 **225** 225) to indicate complete inhibition of hepatic production.



however a qualitative change in the source of the glucose coming from the liver a portion of it is now derived from glucose metabolites coming from insulin sensitive tissues flooding the liver and being reconverted in the liver to glucose. In other words there is a speeding up of the Cori cycle.

LEVINE I should like to show some unpublished data of work which Dr Steele, Dr Bishop and I did at the Brookhaven Laboratory this summer.

There are numerous publications now in the literature on the use of an injection of  $^3\text{C}$  glucose in order to measure the output of  $^3\text{C}$  glucose by the liver. The specific activity of the plasma glucose is determined and the log of the specific activity is plotted against time. The specific activity decreases due to the dilution by  $^3\text{C}$  glucose continuously delivered by the liver. Weinhouse, Wrenshall, Searle and others have shown that the injection of insulin and/or the administration of a single large dose of  $^{14}\text{C}$  glucose stops the further descent of the specific activity curve. This plateau has been interpreted as showing that no further unlabelled glucose is now coming into the pool. Thus glucose and insulin are said to cause an inhibition of glucose output, presumably from the liver.

Dr Steele and Dr Bishop recently did such experiments with one amendment. We used liverless dogs. The animals received a constant injection of  $^3\text{C}$  glucose from a pump so that the  $^3\text{C}$  glucose continuously delivered could not be inhibited by hormonal influences. When unlabelled glucose was given a plateau lasting about 25-30 minutes was obtained. Whatever may be the true explanation it surely cannot be explained by an inhibition of output of glucose by non-existent liver. Insulin did not produce a plateau but neither did it do so in intact controls. It would seem at present that the effect depends upon something present in certain lots of crystalline insulin.

We believe in concert with other experiments by Steele and de Bodo that the glucose plateau is probably due to equilibrium of glucose across body compartments especially between the vascular and extracellular fluid.

KRAHL Stimulation of peptide synthesis by insulin added *in vitro* can be demonstrated in liver slices from mildly diabetic rats (Krahl *Rec. Progress Hormone Research* 1956 12: 199). The stimulation is most marked 2 or 3 days after the induction of diabetes in the liver donor. In long-standing severe diabetes enzymatic mechanisms for peptide synthesis are damaged; insulin then has no effect *in vitro* but restores the capacity for synthesis after injection and the passage of some hours. The *in vitro* insulin effect with liver slices from mildly diabetic rats is related to presence of glucose in the medium; for example the radioactivity of glycine- $^{14}\text{C}$  incorporated into the peptide glutathione was found to be for



control slices from mild diabetics 63. c p m per mg glutathione with insulin but no glucose 598 with glucose but no insulin 1480 with insulin plus glucose 2940

So in summary these are experiments which suggest that under a rather special set of conditions insulin does have an *in vitro* effect on peptide synthesis in liver slices and that this particular effect is due to the effect of insulin on the use of glucose. Later today both Professor Young's group and I will have something to say about the effect of insulin in the absence of glucose on peptide synthesis.

CORR: Thank you Professor Kahl

LONG: What I have to contribute to this subject is negative in the sense that it is not directly concerned with the effect of insulin on liver glycogen. Professor de Duve recalled to your mind that there are other hormones which have a very potent effect in stimulating liver glycogen formation. These of course are the adrenal steroids of the type of cortisone and hydrocortisone.

I think we all appreciate that these materials when given to fasted animals bring about a rapid increase in the amount of glycogen in the liver. I think we should all know and it has been demonstrated many times that this effect is not confined entirely to glycogenesis from the products of increased protein breakdown in the muscles. It has been shown by several investigators including ourselves that if a measured amount of glucose is given to animals with and without the adrenal steroids that in the presence of adrenal steroids a larger proportion of glucose is recovered as liver glycogen. In other words there is a stimulation of glycogen synthesis not only from the intermediary metabolites of protein catabolism but from glucose itself.

The other point that may be mentioned in this regard is the observation made by several laboratories that in severely alloxan-diabetic fasted rats the level of liver glycogen is much higher than that observed in normal rats fasted to a comparable degree. This we have observed and it is of course abolished as many investigators have shown by adrenalectomy.

The point then arises as to whether these effects of the adrenal steroids on liver glycogen formation have any relation to the presence or absence of insulin in the body. There is a certain amount of evidence that they are not related to the presence of insulin. Miller for example has shown that in severely alloxan-diabetic rats which are then adrenalectomized the administration of cortisone to such rats is in the same increase in liver glycogen as is observed in adrenalectomized and have of course are simply adrenalectomized rats.

He concluded, therefore, that this is glycogen in the presence of the

insulin in the presence of the

A good many years ago when Lukens and I were interested in the effect of adrenalectomy on depancreatized animals on some occasions we sacrificed both adrenalectomized-depancreatized animals and hypophysectomized-depancreatized animals at periods of up to 30 or 40 days after the pancreatectomy when they were eating well and presumably in a reasonable state of health and we also found at that time that the amounts of glycogen in the liver were comparable to those found in normal animals subjected to the same dietary regime and of course very much greater than those found in animals that were in diabetic acidosis and coma.

Our general conclusion has been that so far as the stimulation of liver glycogen formation by adrenal cortical hormone is concerned it does not appear to be dependent on the presence of insulin. That of course does not say that insulin is not also required for glycogen synthesis under other circumstances.

CHAIN: I should like to complement Dr Krahls remarks. We have had this experience: we have done a number of perfusion experiments with radioactive glucose in liver with a technique which my colleague Bover has worked out and which gives excellent glycogen synthesis and we find that in normal liver the turnover of glycogen is at maximum so that you could not expect an insulin effect. In fact we do not get one. In the perfused liver of a mild diabetic animal on the other hand we do find an effect on the glycogen synthesis: it is small but it is there.

As far as liver slices are concerned we have now had occasion during our nucleotide work to compare the nucleotides in the intact liver, in the perfused liver and in slices. We find that in the perfused organ about a third of the nucleotides in the intact liver disappear. In the liver slices more than two-thirds disappear. In view of these results we distrust results obtained with liver slices.

I wonder whether this small effect on glycogen synthesis which Professor de Duve has observed on liver slices might not be due to an effect on one particular kind of cell, the Kupffer cells, the phagocytic cells which may be more resistant than normal liver cells to slicing. I think perhaps that could be the explanation and I would be very interested to hear what Professor de Duve has to say about it. We have never been able to find a really significant effect of insulin under those conditions.

CORI: Thank you Professor Chain. Would you like to answer this question Professor de Duve about the Kupffer cell versus the liver cell?

DE DUVE: We have not localized the glycogen in our experiments. This was done a number of years ago by Dr Hastings and his group who found that the glycogen formed by liver slices is deposited almost exclusively in the peripheral layer of cells that is in the cells which receive the most oxygen.

With regard to the other comments made they all sound rather damaging and I feel that if we had shares in the liver most of us would start selling out after what we have heard. There is no doubt that the hepatic actions of insulin are elusive and more easily missed than demonstrated. As a result of this negative results are much easier to obtain than positive ones and the latter are usually registered only by workers who believe an action is to be found and have the patience to go on looking for it under different conditions. Thus personal bias exerts a significant influence on the nature of the results and this is very unfortunate. Despite this fact there are now on record at least ten different instances in which effects of insulin have been observed on the synthesis of glycogen, fatty acids or proteins in liver slices or perfused livers and these have to be explained away as artifacts devoid of physiological significance before the hepatic action of insulin can be denied.

That lack of insulin causes severe disturbances of hepatic metabolism is recognized by everybody and the point at issue is really whether insulin corrects them by a direct and rapid action on the liver or indirectly and in a delayed fashion by way of its effects on other tissues. A well known and important paper by Renold *et al* (*J Biol Chem* 1955 213 135) has done much to further the indirect theory. With Dr Renold's permission, I would like to recall the more recent investigations by Spiro *et al* (*J Biol Chem* 1958 230 761) which argue in favour of the direct theory showing that the hepatic disturbances occur as rapidly as the muscular one in insulin-treated alloxan-diabetic rats following insulin deprivation and that they are then corrected in less than 4 hours by the administration of insulin. Unfortunately the authors did not study the effect of insulin after a shorter time-span.

CORR. This is I think a good summary of the whole problem and I wonder whether Dr Renold would add to what has been said.

RENOLD. Professor de Duve has mentioned the somewhat different results obtained in Dr Hasting's laboratory by Spiro — using insulin-treated alloxan-diabetic rats acutely depleted of insulin — and *et al* — using chronic alloxan-diabetic rats given insulin. The difference for the time being at least is one of degree not quality. Some lag of the liver response when compared with the muscle response was still observed. It is furthermore difficult to know when insulin withdrawal for each tissue really begins. Finally Spiro's findings do not alter the fact that in chronic alloxan-diabetic rats insulin produces hypoglycaemia and increased muscular glycogen synthesis immediately but does not concurrently alter hepatic glucose uptake or output. Hence in this situation at least the hypoglycaemic effect of insulin cannot be secondary to an hepatic effect.

I should also like to comment more generally. Some years ago when

we reported the differences existing in the response to insulin administration of subsequently isolated tissues we were particularly impressed by the differences between tissues rather than with the absence of an insulin effect on liver. We concluded that either insulin did not directly alter liver metabolism or that it exerted a different effect upon this tissue. The need for the differing nature of such an effect upon liver if any has since become even more apparent since the absence of a permeability barrier for glucose in the surface of the liver cell has become so clearly established. Such a different effect of insulin may well exist and it is tempting to suggest that it should perhaps be looked for in the liver as the tissue in which the demonstration of such an effect would be least likely to be clouded by the more easily obtained effect at the cell surface. To be sure the liver cell may have other interphases or membranes than the cell surface. A truly different type of insulin effect upon liver has for instance been recently postulated by a group of workers in Munich (Lamprecht *Biochemische Zeitschrift*, July 1958) although the presently published evidence is not completely convincing.

SMITH: I would like if I may just to ask a question. Liver contains an enzyme which inactivates insulin and which has been called insulinase. Is this a factor which should be taken into consideration in attempts to demonstrate an effect of insulin on carbohydrate metabolism in liver? For example, could the inconstancy of the response of the liver slice to insulin be attributable to destruction of added insulin by this enzyme to variable extents in different experiments?

CORR: Does anyone want to say anything about the destruction of insulin in the liver and how rapidly it might be in normal liver as compared to diabetic liver. Are there any data available? It seems that no one can answer your question. I am sorry.

SCHAMBYE: As you may imagine from my talk I am not willing to sell out any shares to Professor de Duve yet. I think it is very important also to realize the difference between the normal and the diabetic state. When we lack insulin completely certainly the liver glucose metabolism is normal and this may show that ordinarily the insulin present in the normal animal keeps up a normal carbohydrate metabolism which we cannot copy in any way by adding insulin either intraportally or intravenously because we have a different direction of events. Therefore I suppose that before we really give up our shares we should try to study the effect when the liver cells are working in the same way as the muscle cell does when we try to influence it with insulin.

CORR: The amount of work now available on the action of insulin on the liver is large indeed but it seems we need many more experiments before this problem can be finally settled.



PART V

INSULIN AND PROTEIN SYNTHESIS

*Chairman* PROFESSOR F DICKENS



# INSULIN AND INCORPORATION OF AMINO ACIDS INTO PROTEIN

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The original discovery of Von Mering and Minkowski in 1889<sup>17</sup> that the fasting depancreatized dog has a much larger negative nitrogen balance than its normal control has long suggested that insulin has an anabolic influence over protein metabolism. More recently Lukens and McCann<sup>14</sup> have shown that administration of insulin to the depancreatized-hypophysectomized cat leads to the retention of nitrogen and Best *et al.*<sup>11, 12, 13</sup> have found that treatment of the hypophysectomized rat with insulin induces growth though this latter observation has been challenged by Wagner and Scow<sup>23</sup> who claim that insulin only promotes the appetite of the rat and that similar results can be obtained by forced-feeding. Many workers have noticed<sup>5, 7, 13</sup> that insulin lowers the blood amino-acid level of the intact animal and suppresses the rise of blood amino acids which normally follows evisceration.<sup>3, 4</sup> Insulin lowers the blood level of <sup>35</sup>S methionine administered to either the intact or eviscerated dog so that the fall of the blood amino-acid level following insulin is the result of an increased removal of amino acid from the blood rather than a smaller release of amino acid by the tissues. That insulin depresses the rise of the blood amino-acid level in the eviscerated animal indicates that its site of action must be at least in part the extrahepatic tissues.

Lack of a suitable *in vitro* system has so far hampered attempts to disclose in more detail the mechanism of the action of insulin on protein metabolism. In particular experiments so far reported do not differentiate as to whether the effect of insulin on protein metabolism is mediated through a primary action on glucose utilization or by a direct effect on protein metabolism unrelated to the action of insulin on carbohydrate biochemistry. An investigation of this problem has been the aim of the work described here.

Snieszko *et al.* in 1951<sup>21</sup> and Krahf in 1953<sup>2</sup> described experiments in which they studied the incorporation of <sup>14</sup>C alanine and glycine



respectively into the protein of the isolated rat diaphragm when it was incubated in a medium containing the labelled amino acid. Both groups reported that the addition of insulin to the medium stimulated incorporation of  $^{14}\text{C}$  alanine and glycine in a protein when incubated in a medium containing no added glucose or other carbohydrate. Addition to the medium of glucose in place of insulin however produced varied effects. Krahl found that it stimulated the transfer of  $^{14}\text{C}$  glycine into the protein of the diaphragm whereas Simec *et al* found that addition of glucose to the medium actually depressed incorporation of  $^{14}\text{C}$  alanine into protein and that this depression was even more pronounced when pyruvate was added to the medium in place of glucose. Both groups found that addition of glucose or pyruvate to the medium diminished the stimulating effect of insulin on incorporation. In view of the significance of these results it was decided to study further the effect of insulin, glucose and pyruvate on incorporation of a large number of different labelled amino acids into the protein of the isolated diaphragm.

Diaphragm was removed from Albino Wistar rats weighing about 130 g which had fasted about 20 hours before death. The hemi-diaphragms were incubated for 2 hours at  $37^\circ\text{C}$  in a Dubnoff metabolic shaker in a bicarbonate medium gassed with  $\text{O}_2 + \text{CO}_2$  (95:5) and containing labelled amino acid (1 or 3  $\mu\text{mole/ml}$  and specific activity about 130  $\mu\text{C/m-mole}$ ). Glucose and pyruvate when present were added to a concentration of 12.5  $\mu\text{mole/ml}$  (2.5 mg/ml and 1.2 mg/ml respectively). Insulin (Boots crystalline) was used at a final concentration of 0.5 unit/ml. At the end of the incubation the hemi-diaphragms were homogenized in trichloroacetic acid (TCA), washed in hot TCA (to extract nucleic acid), dissolved in NaOH, reprecipitated with TCA, the precipitate washed with alcohol then ether and dried in a vacuum desiccator. The protein samples were ground to a powder, plated and counted as infinitely thick samples. Though no absolute specific activities were determined, the counts obtained from different samples were directly proportional to their different specific activities.<sup>16</sup>

Isolated diaphragm will incorporate  $^{14}\text{C}$  from  $^{14}\text{C}$ -labelled amino acids into protein at a constant rate for a period of at least 6 hours even when no readily oxidizable material is simultaneously added to the medium, but circumstances which interfere with energy production (anaerobiosis and metabolic inhibitors such as cyanide, azide

and dinitrophenol) severely curtail incorporation (Fig. 1).  $^{14}\text{C}$  incorporated into the diaphragm appears to be firmly bound — when diaphragm which has been incubated in a medium containing  $^{14}\text{C}$ -labelled amino acids is incubated for a further period in a similar medium but containing unlabelled amino acids the  $^{14}\text{C}$  in the diaphragm is not lost. It is of interest to note that the rate of incorporation of a  $^{14}\text{C}$ -labelled amino acid is not changed by the addition of other unlabelled amino acids.

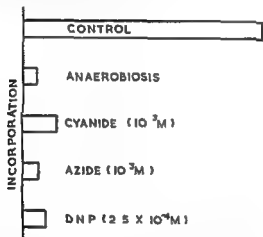


FIG. 1. Effect of anaerobiosis and of the addition of various inhibitors to the medium on the incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$  glycine into protein of the isolated rat diaphragm.

Addition of insulin to a medium containing no added oxidizable substrate has been found to stimulate the incorporation into protein of the isolated diaphragm of ten different  $^{14}\text{C}$ -labelled amino acids of a mixture of  $^{14}\text{C}$ -labelled amino acids (a radioactive protein hydrolysate) and of one  $^{35}\text{S}$  labelled amino acid (Fig. 2). Though the amount of activity incorporated and the magnitude of the stimulation by insulin varied from one amino acid to another, in each case addition of insulin to the medium produced a highly significant stimulation of incorporation of labelled amino acid into protein. The amount of radioactivity incorporated into protein from glutamate and aspartate was rather low, whereas incorporation from leucine in particular was much higher than the general average.

On the other hand the response of leucine to the addition of insulin was rather less than average, whereas the stimulation by insulin of incorporation of methionine was much higher than for any other amino acid

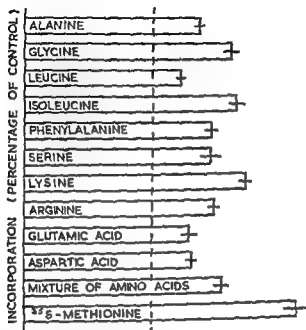


FIG. 2 Effect of the addition of insulin to a medium containing no oxidizable substrate on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of insulin. Incorporation in the absence of insulin is shown by the dotted line. All the amino acids were the L-isomers and uniformly <sup>14</sup>C-labelled except L-methionine and <sup>35</sup>S-methionine.

Addition of glucose to the medium had no observable effect on the incorporation of most of the different amino acids into the protein of isolated diaphragm (Fig. 3) except in the case of methionine, leucine and glutamate where a slight stimulation was obtained, and of alanine where a definite depression of incorporation of <sup>14</sup>C was evident. Addition of pyruvate to the medium likewise had no observable effect on the incorporation of most of the different amino acids into protein of the isolated diaphragm (Fig. 4) except possibly in the case of aspartate and again very definitely in the case of alanine. Alanine apart addition of insulin to a medium already containing

glucose or pyruvate stimulated the incorporation into protein of all the amino acids studied (Figs 5 and 6) and it will be seen by comparison with Fig. 2 that the magnitude of the stimulation produced by insulin is the same when either glucose or pyruvate are added to the medium as in their absence. In general it appears that the incorporation of a labelled amino acid into the protein of the

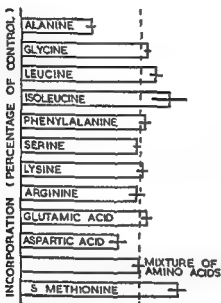


FIG. 5. Effect of the addition of glucose to the medium on the incorporation of radioactivity from labelled amino acid into protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of glucose; incorporation in the absence of glucose shown by the dotted line.

All the amino acids were the L-isomers, and uniformly  $^{14}\text{C}$ -labelled, except  $^{14}\text{C}$  glycine and  $^{35}\text{S}$  methionine.

isolated rat diaphragm is not affected by the addition of glucose or pyruvate to the medium but that it is enhanced by the addition of insulin equally in the presence or absence of added glucose or pyruvate.

These results are interpreted to mean that insulin stimulates protein synthesis as measured by the transfer of labelled amino acids

On the other hand the response of leucine to the addition of insulin was rather less than average whereas the stimulation by insulin of incorporation of methionine was much higher than for any other amino acid

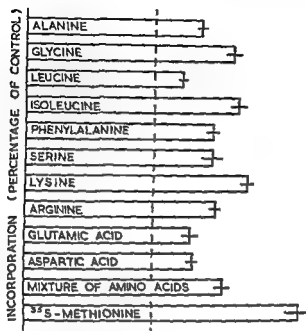


FIG 2 Effect of the addition of insulin to a medium containing no oxidizable substrate on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm

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Alanine apart addition of insulin to a medium already containing

glucose or pyruvate stimulated the incorporation into protein of all the amino acids studied (Figs 5 and 6) and it will be seen by comparison with Fig. 2 that the magnitude of the stimulation produced by insulin is the same when either glucose or pyruvate are added to the medium as in their absence. In general it appears that the incorporation of a labelled amino acid into the protein of the

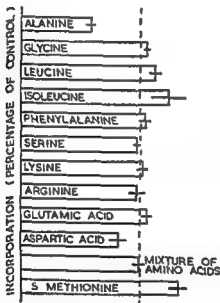


FIG. 3. Effect of the addition of glucose to the medium on the incorporation of radioactivity from labelled amino acids to protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of glucose; incorporation in the absence of glucose is shown by the dotted line.

All the amino acids were the L-isomers and uniformly  $^{14}\text{C}$ -labelled except  $^{14}\text{C}$ -glycine and  $^{35}\text{S}$ -methionine.

isolated rat diaphragm is not affected by the addition of glucose or pyruvate to the medium but that it is enhanced by the addition of insulin equally in the presence or absence of added glucose or pyruvate.

These results are interpreted to mean that insulin stimulates protein synthesis as measured by the transfer of labelled amino acids

from the medium into the protein of the diaphragm by a mechanism that is independent of a stimulation of carbohydrate metabolism. It has been suggested elsewhere<sup>10</sup> that insulin promotes peptide synthesis by increasing the availability of energy and pentoses (for nucleic acids) required in this process. The results presented here provide no support for this view. Nor do they support the thesis<sup>6</sup>

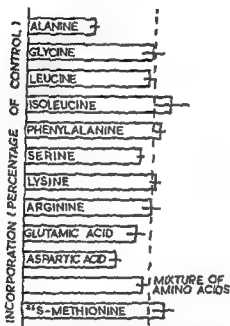


FIG. 4. Effect of the addition of pyruvate to the medium on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of pyruvate; incorporation in the absence of pyruvate is shown by the dotted line.

All the amino acids were the L-isomers and uniformly <sup>14</sup>C-labelled, except 1 <sup>14</sup>C glycine and <sup>35</sup>S methionine.

that a competition may exist between protein and carbohydrate metabolism for the stimulating action of insulin and that stimulation of carbohydrate metabolism by insulin might lead to a diminution of the stimulation by insulin of protein synthesis.

The question remains why the behaviour of alanine is so atypical. Sinek *et al.* considered the possibility that in the presence of added

glucose or pyruvate the incorporation of  $^{14}\text{C}$  alanine into diaphragm protein is diminished because of dilution of the  $^{14}\text{C}$  alanine by unlabelled alanine formed from the added glucose and pyruvate. This possibility has been tested by the addition to the medium of  $^{14}\text{C}$  glucose or pyruvate of the same specific activity as the alanine in

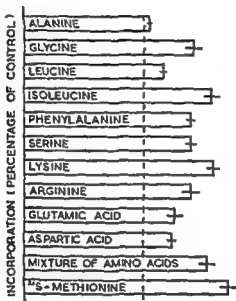


FIG. 5. Effect of the addition of insulin to a medium containing glucose on the incorporation of radioactivity from labelled amino acids into protein of the isolated rat diaphragm.

The blocks represent incorporation in the presence of insulin, incorporation in the absence of insulin is shown by the dotted line.

All the amino acids were the L-isomers and uniformly  $^{14}\text{C}$ -labelled except  $^{14}\text{C}$  glycine and  $^{35}\text{S}$  methionine.

place of unlabelled glucose or pyruvate. Under these circumstances amination of pyruvate to form alanine should not lead to a diminution of the specific activity of the added  $^{14}\text{C}$  alanine. In fact addition of  $^{14}\text{C}$  glucose or pyruvate to the medium in addition to  $^{14}\text{C}$  alanine leads to a greater incorporation of  $^{14}\text{C}$  than when alanine alone is added (Fig. 7) and moreover under these circumstances insulin further stimulates incorporation. These results provide definite



support for the hypothesis that addition of unlabelled glucose or pyruvate depresses the incorporation of  $^{14}\text{C}$  from  $^{14}\text{C}$  alanine into diaphragm protein as a result of the endogenous production of unlabelled alanine<sup>15</sup> Direct evidence that  $^{14}\text{C}$ -labelled amino acids are formed by diaphragm from  $^{14}\text{C}$  glucose and pyruvate is shown in

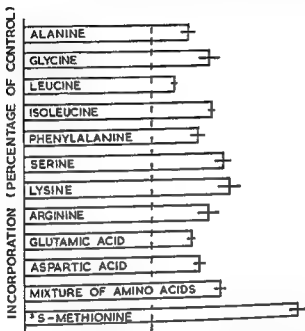


FIG 6 Effect of the addition of insulin to a medium containing pyruvate on the incorporation of radioactivity from  $^{14}\text{C}$  labelled amino acids into protein of the isolated rat diaphragm. The blocks represent incorporation in the presence of insulin incorporation in the absence of insulin is shown by the dotted line. All the amino acids were the L-isomers and uniformly  $^{14}\text{C}$ -labelled except  $^{14}\text{C}$  glycine and  $^{35}\text{S}$  methionine.

Fig 8 A sample of protein from diaphragm incubated with  $^{14}\text{C}$  glucose or pyruvate on hydrolysis and separation by column chromatography into its component amino acids shows peaks of radioactivity for aspartate glutamate and alanine the largest undoubtedly being alanine.

It is pertinent to ask at this stage what is the effect of fasting on the ability of the isolated diaphragm to incorporate amino acids?

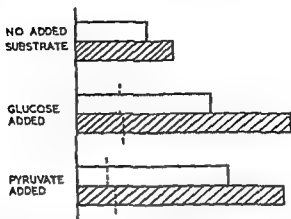


FIG. 7 Effect of addition of  $14\text{C}$  glucose and  $14\text{C}$  pyruvate to medium containing Calne in the incorporation of  $14\text{C}$  into protein of the isolated rat diaphragm. Open blocks represent incorporation in the absence of substrate; hatched blocks in the presence of  $14\text{C}$  substrate.

Calne was uniformly  $14\text{C}$ -labeled pyruvate and di-alanine  $14\text{C}$ . Broken lines indicate the results obtained with unlabeled glucose and pyruvate.

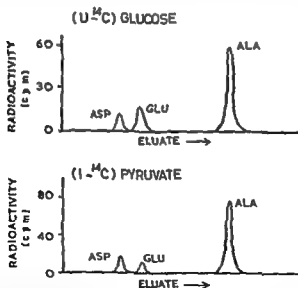


FIG. 8 Location of  $14\text{C}$  in amino acids from protein of isolated rat diaphragm incubated with  $14\text{C}$  glucose or pyruvate.

Amino acids were obtained from diaphragm protein by acid hydrolysis and separated on a Dowex 50 column eluted with  $\text{HCl}$ .

Does lack of food limit the availability of energy and so depress incorporation and the ability of the diaphragm to respond to insulin? The work that has been done so far with diaphragm from 24-hour fasted rats suggests that lack of energy is not rate limiting.

Fig 9 shows the results of an experiment comparing the basal incorporation and response to insulin of diaphragm from the non-fasting 24-hour and 48-hour fasting rat. In fact the basal incorporation is only very slightly if at all affected by fasting and the response

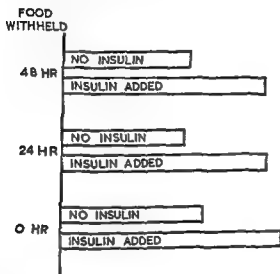


FIG 9 Effect of fasting on incorporation of  $^{14}\text{C}$  glycine into protein of isolated rat diaphragm and its response to insulin (0.1 unit/ml). No glucose was added to the medium.

to insulin is the same in each case. The response of the diaphragm and the effect of insulin on incorporation of amino acids would therefore appear to be as consistent as is the response of the diaphragm and the effect of insulin on glucose uptake.

In the experiments so far discussed insulin has always been used at the rather high concentration of 0.5 unit/ml. This concentration was chosen so that the amount of insulin present should not be a limiting factor. However insulin at very much lower and more physiological concentrations will stimulate incorporation of  $^{14}\text{C}$  glycine into diaphragm protein. Addition to the medium of as little

as 0.5 milliunit of insulin/ml produces a half-maximal stimulation of incorporation with or without addition of glucose to the medium (Fig. 10) and a smaller but still statistically significant stimulation is observed with as little as 0.05 milliunit/ml. Addition of insulin to a concentration of 0.01 milliunit/ml has no observable effect. Even at these concentrations there is no suggestion that addition of glucose

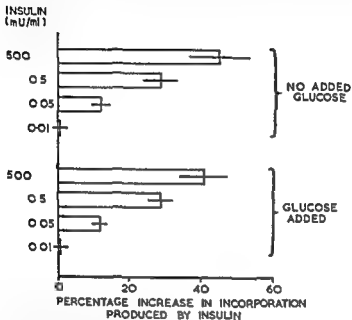


FIG. 10. Comparison of the effect of insulin at low concentrations on the incorporation of  $^{14}\text{C}$  glycine into protein of the isolated rat diaphragm incubated in media with and without added glucose.

to the medium appreciably alters the response of the isolated diaphragm to the stimulating effect of insulin on the incorporation of  $^{14}\text{C}$  glycine into protein.

Finally, administration of insulin to the normal rat stimulates the incorporation of  $^{14}\text{C}$  glycine into the protein of the subsequently isolated diaphragm (Fig. 11) whereas incorporation of  $^{14}\text{C}$  glycine into protein of the diaphragm from the alloxan-diabetic rat is barely half that of the diaphragm from the normal rat. Treatment of the

alloxan-diabetic rat with insulin raises the ability of its isolated diaphragm to incorporate  $^{14}\text{C}$  glycine into its protein at nearly the normal rate

To summarize the results show clearly that insulin at several concentrations and under a variety of conditions stimulates the incorporation of amino acids into the protein of the rat diaphragm. These experiments however do not indicate the actual mechanism by which insulin brings about this stimulation though it appears to be independent of a simultaneous augmentation of glucose utilization

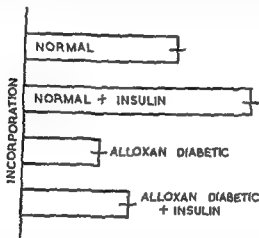


FIG. 11. Effect of alloxan diabetes and of treatment with insulin of the normal and alloxan diabetic rat on the incorporation of  $^{14}\text{C}$  glycine into protein of the isolated diaphragm. Normal rats treated with insulin received 1 unit subcutaneously 1 hour before death. Alloxan-diabetic rats received 1 unit PZ insulin daily for 4 days before death.

because neither the basal incorporation nor the enhancement of incorporation by insulin is affected by the addition of glucose or pyruvate to the medium. Insulin could stimulate the transfer of labelled amino acids into the protein of diaphragm in at least two ways either by enhancing the movement of amino acids from the medium into the cells of the tissue or by promoting the activity of the protein synthesizing mechanism within the cell. In view of the experiments of Levine and Goldstein<sup>12</sup> and of Park *et al*.<sup>13, 14</sup> on the effect of insulin on the transport of sugars into muscle it seems likely that insulin enhances the entry of amino acids into the cell. This

view would be in accord with the recent findings of Kipnis and Noall<sup>18</sup> that insulin stimulates the transfer of amino-isobutyric acid from the medium into the tissue of the whole diaphragm preparation. However the actual mechanism by which insulin brings about this occurrence still remains obscure

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# HORMONES AND PROTEIN SYNTHESIS

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A cell free system can be prepared from rat liver which will incorporate radioactive amino acids into protein *in vitro* (Sickevitz<sup>19</sup> Zamecnik and Keller<sup>23</sup> Sachs<sup>16</sup>). In its essentials the system is a simple one. Rat liver is lightly homogenized at 0° in a suitable buffered medium containing salts and the unbroken cells, cell debris, nuclei and mitochondria are removed by centrifugation. The supernatant which contains the microsomes and the soluble fraction of the cell is shaken at 37° with adenosinetriphosphate (ATP) as energy source; an ATP generating system (phosphocreatine and phosphocreatine kinase) were used in the experiments to be described) and guanosine triphosphate (GTP). When a radioactive amino acid is added under these conditions some of it is incorporated into protein.

I have been using preparations of this type as a test system in an investigation of the way in which hormones influence the biosynthesis of proteins. In doing so there is an implicit assumption that the reactions studied with this cell-free system are those which are occurring during protein biosynthesis *in vivo*. No one has yet shown that net synthesis of protein occurs in this *in vitro* system but this is not surprising in view of the short life of the cell-free system. Evidence is accumulating however which strongly supports the view that the reactions which occur during amino-acid incorporation into protein *in vitro* are also the reactions which occur during protein synthesis *in vivo*. It is known for instance that when amino acids are incorporated into protein in this system they are located throughout the length of the peptide chain not just at its ends and the newly incorporated radioactive amino acids are joined to other amino acids by  $\alpha$ -peptide bonds. It is unlikely that the reaction studied with the *in vitro* system is an exchange between the labelled amino acid supplied and a similar unlabelled amino acid in protein because labelled amino acids once they are incorporated are not washed out of protein by incubation with excess unlabelled amino acid. The view that protein synthesis is



occurring albeit at a very slow rate is also supported by the observation that more amino acid is incorporated into protein when several radioactive amino acids are added to the incubation mixture than when only one labelled amino acid with the same amount of radioactivity is present, for this suggests that several amino acids are simultaneously incorporated into protein as one would expect to occur during protein synthesis. Finally Campbell *et al*<sup>2</sup> have

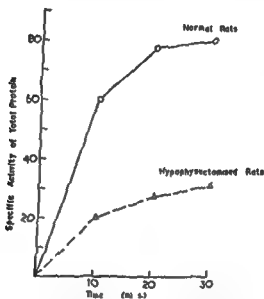


FIG 1 Specific activity of protein after various time of incorporation of DL-leucine-<sup>14</sup>C in a cell free system. Comparison of normal and hypophysectomized rats

shown that much of the radioactive amino acid incorporated into protein in this system goes into a specific protein namely albumin. All of these observations encourage one to believe that the events observed with the *in vitro* system do reflect something of the events which occur during protein synthesis in the cell but one must of course be on one's guard to detect distorted reflections of these events.

I started this work (Korner<sup>10</sup>) by comparing the amounts of radioactive amino acid incorporated into protein in systems of this type prepared under rigidly standardized conditions from the livers of normal rats and from rats which had been hypophysectomized.

Both groups of rats ate the same ration of food. Fig. 1 shows that the amino acid is incorporated rapidly into proteins of the liver preparation from normal rats and that the specific activity of the protein reaches a plateau after about 20 minutes. A similarly shaped curve was drawn from results obtained with a preparation from the liver

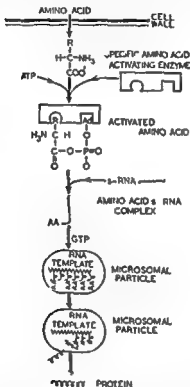


FIG. 1 Schematic representation of the present-day hypothesis of the sequence of reactions in protein biosynthesis.

of hypophysectomized rats but here the final specific activity of the protein was only about 40 per cent of that attained by the protein from the normal rats.

One can now pose the question: Where among the many steps which are known to occur during the biosynthesis of proteins are the pituitary hormones exerting their influence? Fig. 2 summarizes

in highly schematic form, the steps which are thought to occur during protein biosynthesis. Some of these steps have been quite well established on some points the evidence is not conclusive and occasionally there is frank speculation but the whole presents a reasonable working hypothesis which enables one to design experiments and which can be altered or abandoned as necessary. This scheme takes no account of the protein synthesis which occurs in nuclei (Allfrey *et al*<sup>21</sup>) or mitochondria (Simpson *et al*<sup>21</sup>) since little detail is known of the sequence of reactions involved in these systems.

Amino acids cross the cell wall probably by some active process (or are synthesized inside the cell) and are activated by specific amino-acid activating enzymes (Hoagland *et al*<sup>6</sup>). It is supposed that there are twenty enzymes of this type in the cell sap each specific for one amino acid although the existence of some of the twenty has yet to be demonstrated (Lipmann<sup>11</sup> Nisemann *et al*<sup>13</sup>). The activation step requires ATP and it is suggested that an amino acyl-adenosine-monophosphate complex is formed on the surface of the activating enzyme. It is possible that Vitamin B<sub>12</sub> may be a co-factor of the activating enzymes (Wagle *et al*<sup>22</sup>). Hoagland *et al*<sup>6</sup> have good evidence that the activated amino acid now forms a complex with the low molecular weight (10 000-20 000) soluble ribonucleic acid (s-RNA) of the cell. The amino acid is attached to the polynucleotide through adenosine and cytosine monophosphates (Hecht *et al*<sup>6</sup>) and it is possible that more than one subfraction of the s-RNA exists with varying affinities for each amino acid (Schweet *et al*<sup>16</sup>). GTP is needed in the next step which is the transfer of the amino acid from the s-RNA (Hoagland *et al*<sup>7</sup>) to the RNA template of the microsomal particles where it falls into a place on the template which is specific for that amino acid. Crick<sup>3</sup> has suggested that each amino acid may be bound to a specific trinucleotide of the s-RNA and that it is this trinucleotide with the amino acid attached which enters the microsomal particle and binds to the complementary bases on the RNA template by base pairing thus placing the amino acid in a specific position. It is possible to construct schemes which will satisfactorily account for the way in which the four bases of RNA taken three at a time can code without ambiguity the twenty amino acids found in proteins (Crick *et al*<sup>4</sup>).

Littlefield *et al*<sup>12</sup> have produced good evidence that the actual assembly of polypeptide chains from amino acids occurs in the microsomal particles. These particles are ribonucleoprotein

granules about 100-150 Å in diameter and they are probably identical with the electron dense granules noted by Palade<sup>15</sup> on one side of the lipoprotein membrane of the endoplasmic reticulum in rat liver and other cells. When all the amino acids are present on their specific loci on the RNA template in the microsomal particles peptide bond formation occurs between them and the finished peptide with its specific amino-acid sequence peels off the template and passes first into the rest of the cell via the endoplasmic reticulum and if the cell is a secretory one into the organism as a whole.

TABLE I

EFFECT OF HYPOPHYSECTOMY ON THE INCORPORATION OF LABELLED AMINO ACIDS INTO MICROSOMAL PROTEIN IN VITRO  
(c.p.m./mg.)

Soluble		Microsomes from		
		Normal rat	Hypophysectomized rat	
Fraction	Normal rats	146	87	Ns
	Hypophysectomized rats	138	76	Hs
		Nm	Hm	

One could study each of these steps separately in an attempt to identify the point or points at which pituitary hormones are exerting their effects but a short-cut is possible. Centrifugation of the cell free system at 105 000 g. separates the microsomes from the soluble fraction of the cell which contains the activating enzymes and the s-RNA intermediate. Neither of these two parts of the cell incorporated much radioactive amino acid alone but together they incorporate as much as the unseparated system. It is possible that one of these two parts of the whole system is unaffected by hypophysectomy. Table I summarizes in matrix form the results of an experiment in which microsomes and soluble fractions

separated from the livers of normal and hypophysectomized rats. When microsomes from normal rats (Nm) were incubated under the conditions described with soluble fraction (Ns) from normal rats the specific activity of the microsomal protein was 146 c.p.m./mg but when microsomes (Hm) and soluble fraction (Hs) both from hypophysectomized rats were incubated together under identical conditions only about half as much amino acid was incorporated into protein. If microsomes from hypophysectomized rats (Hm) are incubated with soluble fraction from normal rats (Ns) there is only a small rise in the amount of amino acid incorporated into protein compared with that obtained when the microsomes from hypophysectomized rats (Hm) were incubated with soluble fraction, also from hypophysectomized rats (Hs). Again when microsomes from normal rats (Nm) are incubated with soluble fraction from hypophysectomized rats (Hs) almost as much amino acid is incorporated into microsomal protein as when the microsomes from normal rats (Nm) are incubated with soluble fraction from normal rats (Ns). This must mean that hypophysectomy has not within the limits of these experiments resulted in a significant change in the ability of the activating enzymes to activate amino acids nor in the ability of the s-RNA to form an amino-acid complex or to transfer the amino acid from the s-RNA complex to the microsomes. The lower incorporation of amino acids into protein shown by the system prepared from hypophysectomized rats compared with that from normal rats must be a result of some change in the microsomes themselves.

It might be argued that microsomes from hypophysectomized rats suffer more extensive degradation during the course of an experiment than the microsomes from normal rats and that this accounts for the lower incorporation shown by microsomes from hypophysectomized rats. This could be caused either by the presence of more active degradative enzymes such as cathepsins or ribonuclease in the preparation from hypophysectomized rats or by some alteration in the microsomes as a result of hypophysectomy which makes them more susceptible to these enzymic activities. Experiments were therefore carried out in which the incorporation of radioactive leucine into microsomal protein was measured after various periods of pre-incubation of the microsomes either in the presence of the whole homogenate or in the presence of the soluble fraction of the cell. Fig. 3 shows the results obtained in one such

experiment when microsomes were pre-incubated alone and it is clear that pre-incubation does depress the ability of the microsomes to incorporate amino acids into protein but the effect of pre-incubation on microsomes from hypophysectomized rats and on those from normal rats is very similar. This result supports the view that the change in the microsomes which occurs as a result of hypophysectomy is one which depresses the ability of the microsomes to incorporate amino acids in the animal and is related to the

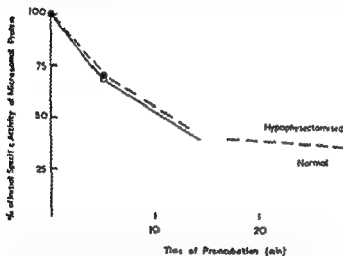


Fig. 1. Effect of various times of pre-incubation of a microsome fraction from normal hypophysectomized rats on the specific activity of their protein after 30-min post-incubation. The results are expressed as per cent of the specific activity after a 30-min incubation in an experiment in which the microsome fraction had not been pre-incubated.

depression of protein synthesis in the liver which hypophysectomy (Simpson *et al.*<sup>20</sup>) and is not a secondary effect which becomes apparent only as a result of the experimental techniques used.

Fig. 4 shows the results obtained when the microsome fraction was separated again from the soluble fraction after incubation for 30 minutes and the radioactivity of each protein fraction determined. It is clear that hypophysectomy of the rat significantly altered the ability of the synthesized  $\frac{1}{2}$  to  $\frac{1}{4}$  off the RNA template of the microsomes since the

activity in the soluble fraction protein to that in the microsomal protein is similar in the preparations from normal and hypophysectomized rats

It was found that microsomes from hypophysectomized rats have less RNA per mg protein than microsomes from normal rats. However the differences in the ability to incorporate amino acids into protein cannot be ascribed simply to a quantitative change in the RNA of the microsomes brought about by hypophysectomy for as is shown in Fig. 5 a qualitative change has occurred in the

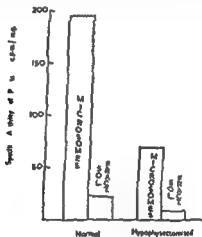


Fig. 4 Specific activity of microsomal protein and of soluble fraction protein from normal and hypophysectomized rat liver separated again after incubation for 30 minutes *in vitro* with L-leucine-<sup>14</sup>C

RNA of the microsomes as a result of hypophysectomy. The specific activity of microsomal protein is plotted against the  $\mu\text{g}$  of RNA present in the microsomes in Fig. 5 and it is clear that the RNA of microsomes from hypophysectomized rats is less efficient on a weight for weight basis than RNA from microsomes from normal rats at incorporating amino acid into protein. It is also clear from Fig. 5 that addition of microsomal RNA from normal rat liver results in the maintenance of the specific activity of microsomal protein at a steady rate but a similar addition of microsomal RNA from liver of hypophysectomized rats results in a steady fall in microsomal specific activity. That part of the RNA of the microsomes which is particularly concerned in protein biosynthesis is

altered as a result of hypophysectomy so that it is less capable of acting as a template. When microsomes from hypophysectomized rats are added to the system one is adding partly inactive RNA.

Experiments have been and are being carried out to see if treatment of the hypophysectomized rats with pituitary and other hormones can restore the ability of microsomes from hypophysectomized rats to incorporate amino acids into protein at a normal rate. The results obtained with two hormones GH and insulin

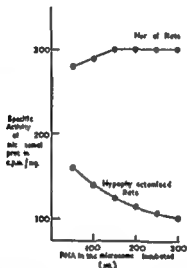


FIG. 5. Specific activity of microsome protein from normal and hypophysectomized rats after a 30-min incubation period with varying amounts of microsomal RNA.

will be mentioned. It was found that treatment of hypophysectomized rats with ox GH was successful in markedly raising the amount of amino acid which is incorporated into protein in the *in vitro* system and it was shown that GH acts mainly by improving the incorporating ability of the microsomes and not on the reactions carried out in the soluble fraction of the cell. In this respect GH is reversing the effects of hypophysectomy but full restoration of the incorporating ability of the microsomes to the normal level has not been achieved by treatment with GH alone. It is probable that the



other pituitary hormones play some part in the control of the reactions which bring about protein synthesis

The anabolic action of insulin can also be demonstrated with this system. Table II is a matrix diagram which shows that when hypophysectomized rats are treated with insulin (0.5 units PZI/day for 7 days) almost three times as much amino acid is incorporated

TABLE II  
EFFECT OF TREATMENT OF HYPOPHYSECTOMIZED RATS WITH INSULIN (0.5 U PZI/DAY FOR 7 DAYS) ON THE INCORPORATION OF LEUCINE  $^{14}\text{C}$  INTO MICROSMAL PROTEIN *IN VITRO*  
(cpm/mg)

	Microsomes from		
	Hypophysectomized rats	Insulin treated hypophysectomized rats	
Hypophysectomized rats	25	59	H <sub>1</sub>
Insulin-treated hypophysectomized rats	36	73	H <sub>1</sub> I <sub>2</sub>
	H <sub>1</sub> II <sub>1</sub>	H <sub>1</sub> II <sub>2</sub>	

into protein in *in vitro* experiments compared with that incorporated into the protein from untreated hypophysectomized rats. In this case not only were the microsomes from insulin-treated rats able to incorporate more activated amino acids than those from untreated rats but in addition the soluble fraction of the cell was better able to prepare the radioactive amino acid for incorporation. This is shown by the fact that incubation of the microsomes from hypophysectomized rats (H<sub>1</sub>) with soluble fraction from insulin treated hypophysectomized rats (H<sub>1</sub>I<sub>2</sub>) resulted in greater incorporation of amino acid than occurred when the microsomes and soluble fraction were both from untreated hypophysectomized rats.

Table III summarizes the results of preliminary experiments with rats made diabetic with alloxan. The lower incorporation of amino

acids into protein shown by the alloxan-diabetic rats (D) compared with control rats (N) is somewhat restored when the diabetic rats were treated with insulin (DI) but not altered significantly when they are treated with  $\alpha$  GH (DG). These observations lend support to the view (see Ketterer *et al*<sup>9</sup> for discussion of this subject) that GH requires the presence of insulin before its anabolic action can be fully realized but that insulin can cause anabolism in the absence of GH (Salter and Best<sup>17</sup>).

TABLE III

EFFECT OF ALLOXAN DIABETES (D) AND OF TREATMENT OF ALLOXAN-DIABETIC RATS WITH INSULIN (2 LU PZ/DAY FOR 5 DAYS) (DI) OR GROWTH HORMONE (0.2 MC/DAY FOR 5 DAYS) (DG) ON THE INCORPORATION OF LUCINE-<sup>14</sup>C INTO MICROSOMAL PROTEIN *IN VITRO* (c.p.m./mg.)

		Microsomes from			
		Normal rats	Diabetic rats	Insulin-treated diabetic rats	GH-treated diabetic rats
Soluble fraction	Normal rats	94	77	89	74
	Diabetic rats	79	66		
	Insulin-treated diabetic rats	87		89	
	GH-treated diabetic rats	81			72
		N <sub>1</sub>	Dm	DIm	DGIm

Finally, I want to mention the results of some experiments on the effect of hormones on the incorporation of amino acids into protein *in vivo* which are in agreement with the conclusions drawn from the *in vitro* experiments which have been discussed. In these experiments amino acids were injected into the femoral vein of the rats and successive lobes of the liver removed at 10 and 20 minutes after the injection. Each lobe of liver was homogenized and the soluble fraction and microsomes separated from one another. Microsomes were treated with sodium deoxycholate as described by Littlefield *et al*<sup>12</sup> and separated into two microsomal particles containing about 50 per cent R<sub>1</sub>.

cent protein (Palade and Siekevitz<sup>15</sup>) and the fraction probably consisting of the lipoprotein endoculum. The radioactivity of the proteins of each of fractions was assessed and are plotted in Fig 3 which shows amino acid is rapidly incorporated into protein in the particles and that the radioactive protein is transferred to deoxycholate soluble fraction of the microsomes and to soluble fraction of the cell (cf Littlefield *et al*<sup>12</sup>)

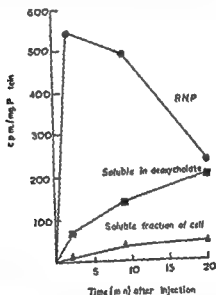


FIG 6 Specific activity of protein of microsomal particles (—●—) fraction soluble in sodium deoxycholate (—■—) and the soluble fraction of the cell (—▲—) at various times after intravenous injection of radioactive amino acid into a normal rat.

In hypophysectomized rats less amino acid is incorporated into protein in the microsomal particles than in normal rats and in sequence less radioactive protein is transferred to the deoxycholate soluble fraction of the microsomes and to the soluble fraction of the cell. Treatment of the hypophysectomized rats with ox GH or with insulin increased the incorporation of amino acids into protein in the microsomal particles (Fig 7) and consequently increased the specific activity of the other two fractions.

## SUMMARY

Hypophysectomy reduces the amount of amino acid which is incorporated into the proteins of liver *in vivo* or *in vitro*. The activity of the amino-acid activating enzymes is not significantly altered by hypophysectomy nor is the ability of the s-RNA to form an amino-acid-s-RNA complex. The microsomes themselves are

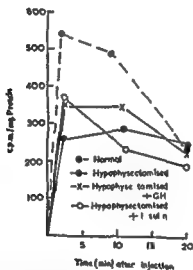


Fig. 7. Specific activity of microsomal particle protein from normal, hypophysectomized, GH-treated hypophysectomized and insulin-treated hypophysectomized rats at various times after intravenous injection of radioactive amino acid.

less able to incorporate amino acids into protein as a result of hypophysectomy probably because of a change in the RNA of the template. Treatment of hypophysectomized rats with  $\alpha$ -growth hormone or with insulin could restore in part the impaired ability of the microsomes to incorporate amino acids into protein. Alloxan-diabetic rats showed a similar impaired amino-acid incorporating ability which could be restored by treatment of the diabetic rats with insulin but not under the conditions described by treatment with  $\alpha$ -growth hormone.

## ACKNOWLEDGMENTS

I wish to thank the Council of the Royal Society for a Grant in aid Miss M B Thomas who performed the hypophysectomies and Mr D C Gardiner for highly skilled assistance It is a pleasure to thank Professor F G Young MRS for his interest in this work and for his encouragement

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## DISCUSSION

KRAHL For some years it has been clear that insulin has both short term and long term effects on protein synthesis. In these two extremely lucid papers from Professor Young's laboratory both aspects of the effect of insulin on protein synthesis have been discussed. The second paper by Dr Korner opens up new avenues of experiment regarding the long-term effects of insulin on protein synthetic processes. The remainder of my discussion will be devoted to describing some experiments of our own which cover somewhat the same ground as those discussed by Dr Manchester with a slightly different point of view.

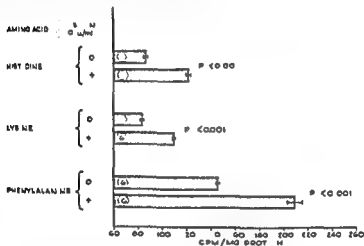


FIG. 1. Incorporation of  $C^{14}$  Amino acids into muscle protein. Effect of insulin in the absence of glucose.

My own interest in this, as pointed out by Dr Manchester, began in 1953, when Sinex, Hastings and MacMullen on the one hand and I on the other independently observed and reported that insulin apparently had an effect to stimulate amino-acid incorporation into protein in the absence of added extracellular glucose. This effect of insulin, which is independent of that on glucose transport, apparently represents one facet of the general action of insulin to initiate cellular rearrangements favouring anabolism (Krahl *Perspectives Biol. and Med.* 1957 1: 69). Two years ago my colleague Dr Wool began further experiments in this field to establish the reality of this effect of insulin in the absence of glucose; his results have been remarkably similar to those described by Manchester. I will review them rapidly to show that in one field, at any rate two

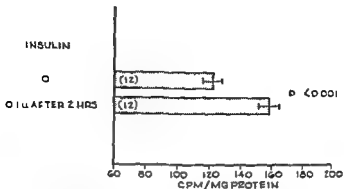


FIG 2 Incorporation of  $C^{14}$  Histidine into muscle protein. Effect of insulin added after 2 hours prior incubation — no glucose present

independent groups of investigators have got exactly the same or nearly the same results. This is extremely rare in anything having to do with the mechanism of insulin action.

In diaphragms from *f d* rats insulin stimulates incorporation of histidine, lysine or phenylalanine into protein *even in the absence of added glucose*.

In an effort to reduce the amount of glucose which might have been

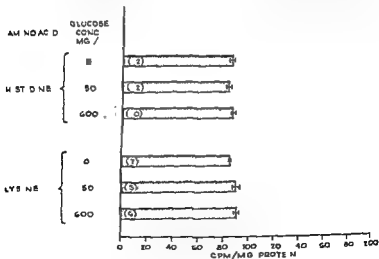


FIG 3 Incorporation of  $C^{14}$  Amino acids into muscle protein. Effect of glucose concentration

carried over by the diaphragms from the fed rats we incubated the diaphragms for  $\approx$  hours without glucose or insulin then added insulin to half the samples and continued the incubation for  $\approx$  hours more. Under these conditions in which any glucose in the extracellular fluid would be exhausted we still observed a stimulation by insulin of amino-acid incorporation.

These are further control experiments with diaphragms from *fed* rats. The argument was that if the effects of insulin described above were due to increased use of glucose under the stimulus of insulin then addition of enough glucose would produce the same effect as insulin. It had been

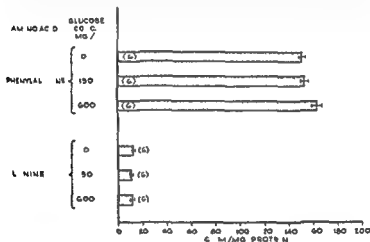


FIG. 4. Incorporation of L-phenylalanine and L-leucine into muscle protein. Effect of glucose concentration.

previously shown by Gemmell years ago and by us under the present experimental conditions, that increase in glucose concentration led to an increase in glucose use. However as you see the addition to the medium of 150 mg per cent or even 600 mg per cent glucose did not promote incorporation of histidine, lysine, phenylalanine or alanine into protein of diaphragms from *fed* rats. We therefore concluded that the effect of insulin shown in Figs. 1 and 2 was in itself one concerned with amino acids *per se* and not with glucose transport.

The original experiments undertaken by Wool were concerned simply with establishing an effect of insulin on amino-acid incorporation in the absence of glucose. By the time this had been completed other questions



arose what is the mechanism of this effect of insulin in the absence of glucose? In particular is this effect of insulin completely accounted for by an effect on the transport or accumulation of amino acids? The present answer to this second question appears to be negative for reasons stated below

Kipnis and Noall (*Biochim Biophys Acta* 1958 28 226) working at Washington University reported that insulin increased the rate of accumulation of a non utilizable amino acid  $\alpha$  aminoisobutyric acid (AIB) by diaphragms *in vitro*. There are differences between the effect of insulin on AIB accumulation and on amino-acid incorporation into protein (Fig 5)

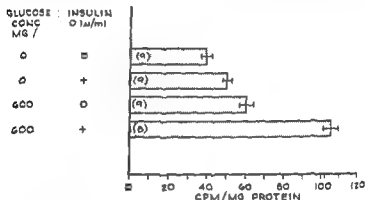


Fig 5 Incorporation of  $C^{14}$  Histidine into muscle protein  
Effect of glucose and insulin 48 hours fasted rats

First insulin promotes AIB accumulation but not significantly the incorporation of amino acid into protein by diaphragms from animals fasted for 2 or 3 days. This demonstrates clearly that an increase in radioactivity of the protein does not necessarily follow an increase in the specific radioactivity of the amino acids of the intracellular pool. Secondly with diaphragms from fasting animals addition of glucose and insulin to the incubation medium gives no greater stimulation of AIB accumulation than insulin alone. In contrast glucose and insulin act synergistically upon incorporation of amino acid into protein giving a stimulation larger than the sum of the stimuli produced by either alone. From these facts Wool and I have reached the working hypothesis that insulin may have some effect on peptide synthesis in addition to that on amino-acid transport.

# A. KORNER

In summary insulin promotes peptide synthesis in rat muscle. Two components of the insulin effect can be distinguished from fed rats: insulin stimulates incorporation of amino acids into protein even in absence of added glucose. Whether this is accounted for by an insulin effect on amino-acid transport from extracellular to cellular phase remains to be settled by further experiment. In diaphragm from 48 hour fasting rats insulin alone has no significant effect on protein synthesis; glucose alone promotes it, and insulin and glucose together synergistically. A similar situation obtains for synthesis of protein in liver slices from mildly diabetic rats. This component of insulin anabolic action is apparently dependent on stimulation of glucose utilization.

In conclusion, I should like to mention that Professor H. Cl. and co-workers have shown that other hormones do have effects on amino acid accumulation by animal tissues. For example, after injection, adrenal C 11 oestrogens promote accumulation of AIB by liver but not by certain other tissues; this may be related to the conversion of protein to glycogen. Also oestrogen promotes accumulation of AIB by uterus but not by other tissues such as liver upon which oestrogen has no particular growth-promoting effects. So we are now in the position of having to unravel the contributions of individual hormones by way of amino-acid transport and to show whether or not these various hormones have still other effects on protein synthesis.

DICKENS: Thank you very much Professor Kahl for your interesting criticism and confirmation of some of the work of the preceding speakers. It is obvious to us all now, I think, that there is room for alternative hypotheses on the action of insulin on the incorporation of amino acids as in most other fields concerning insulin action. The balance of evidence seems to be in favour of direct stimulatory effect of insulin on amino-acid incorporation which is apparently independent of glucose transport, as we find Professor Kahl and Dr Manchester in agreement on this basic point.

There is also the question of the extent of energy required for the protein synthesis which bears upon the work of Dr Korner who has in a very original way, I think, applied the findings of Lipmann and Zamecnik and others on modern views of protein biosynthesis to try to localize the exact point of action and if I understood it rightly, tends to pin this down to an alteration in the effectiveness of ribonucleic acid in promoting protein incorporation in the cell.

So we have had at least two views which are perhaps not easy to reconcile but in these matters there is more than one factor affected, and this is a new situation.

PETERSON: May I make two little points. The first is did Dr Korner

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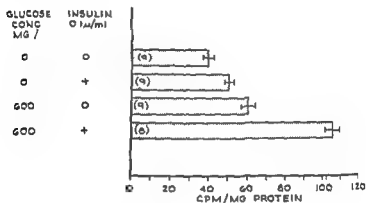


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In summary insulin promotes peptide synthesis in rat diaphragm muscle. Two components of the insulin effect can be identified. In diaphragms from fed rats insulin stimulates incorporation of amino acids into protein even in absence of added glucose. Whether this is accounted for by an insulin effect on amino-acid transport from extracellular to intracellular phase remains to be settled by further experiment. In diaphragms from 48 hour fasting rats insulin alone has no significant effect on peptide synthesis. Glucose alone promotes it and insulin and glucose together act synergistically. A similar situation obtains for synthesis of glutathione or protein in liver slices from mildly diabetic rats. This component of insulin anabolic action is apparently dependent on stimulation of glucose utilization.

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DICKENS: Thank you very much Professor Krahl for your interesting criticism and confirmation of some of the work of the preceding speakers. It is obvious to us all now I think that there is room for alternative hypotheses on the action of insulin on the incorporation of amino acids as in most other fields concerning insulin action. The balance of evidence seems to be in favour of direct stimulatory effect of insulin on amino-acid incorporation which is apparently independent of glucose transport as we find Professor Krahl and Dr Manchester in agreement on this basic point.

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So we have had at least two views which are perhaps not easy to reconcile but in these matters there is more than one factor affected and this is no new situation.

PETROW: May I make two little points? The first is did Dr Korner

use rats that were adult? Because as hypophysectomy alters the pattern I wondered whether castration would alter the pattern. There may after all be some sort of synergistic effect between the testosterone type of compound and insulin and that brings me to the next point.

It has been suggested recently — I think in the *BMJ* within the last 2 years — that insulin is the *major anabolic hormone* of the body. Now that may well be true and I think probably prior to puberty it is difficult to see where the anabolic effect is going to come from. There is no testosterone present as far as we know in the tissues. But after puberty you have I think a synergistic effect between androgens and the insulin hormone.

KORNER In answer to the specific points raised by Dr Petrow — (a) The rats used in the experiment I have described were young adults i.e. 3-5 months of age and weighing 150-200 gms. (b) Preliminary experiments with castrated male rats indicate that androgens are concerned in the amino-acid incorporation into protein in the system I have described.

YOUNG I did not understand clearly whether Dr Petrow was suggesting that the growth hormone of the pituitary gland was not an important anabolic hormone of the body. I understood him to say that insulin was the major one. Did I understand correctly?

PETROW That is the suggestion put forward in this recent paper.

YOUNG I do not think that we can dismiss the effect of the pituitary gland on growth as lightly as that. I myself am very impressed by the importance of the pancreas in stimulating anabolism of protein but the work of Scow in recent years has shown that in the rat at any rate one sees an anabolic effect of growth hormone in completely depancreatized animals maintained on a constant dosage of insulin. While one should not underrate the importance of insulin in anabolic processes such as protein synthesis I think one also should not be so much swayed by such ideas as to demote the pituitary gland in general and its growth hormone in particular.

LONG I would like to address a question to Dr Korner. Since removal of the hypophysis removes two types of effects on protein metabolism the growth hormone as well as the adrenal corticotrophic hormone has he studied these effects in animals deprived of their adrenals or previously treated with large amounts of ACTH or hydrocortisone? One might anticipate rather opposite effects with ACTH and growth hormone.

KORNER Thank you Professor Long for your question on the role of the adrenals in this amino-acid incorporating system. My preliminary observations show that small doses of ACTH given to normal or hypophysectomized rats decrease the amino-acid incorporation into protein obtained with the microsomal system but that when small amounts of ACTH are given to hypophysectomized rats together with GH a some-

what greater incorporation is seen than when GH is given alone. I may add that adrenalectomy results in decreased incorporation of amino acids into protein *in vitro* and that the effect is exerted both on the microsomal and on the soluble fraction of the cell.

DALE. I am not at all sure that my intervention is properly relevant to the brilliant contributions to this symposium which we are supposed now to be discussing and which have been concerned with the evidence for the promotion by insulin of the synthesis of cell proteins from amino acids and with the more detailed method of that synthesis. Listening to these communications however and more especially to some of the earlier and equally important ones dealing with the effect of insulin on the relation between the glucose contents of the blood as it enters and as it leaves the liver in the living animal I have been wondering whether in discussing the significance of the positive and negative balance observed sufficient attention has in all cases been given to both sides of the account. Most of you will be aware that I have never had any proper ground for a claim to be regarded as a carbohydrate biochemist or indeed as a real biochemist of any kind. I cannot help recalling however the experiences which I had in the very early days of research on insulin and its actions in as happy and lively a collaboration as it was ever my privilege to enjoy with Charles Best whom we would so gladly have seen here today. Joseph P. Hoet who is actually here and my own colleague at that time the late H. P. Marks. Students of the effects of insulin were even then faced with what seemed to be the paradox that insulin as was to be expected caused glucose to disappear from the circulatory blood, but that nobody had been able to discover what became of it. The first and natural supposition had been that it would have been synthesized to glycogen and deposited as such in the tissues. When however an excessive dose of insulin was injected into normal mice with the result that they fell into hypoglycaemic coma in some 20 to 30 minutes so far from the glucose which disappeared from the blood being discoverable anywhere in the body as extra glycogen all the naturally occurring glycogen of the liver muscles or any other tissue had also disappeared leaving an animal practically devoid of free carbohydrate in any recognizable form. The theory which was then rather freely promulgated indeed was that insulin caused the transformation of carbohydrate into some unknown form in which it escaped detection by available methods. With my then still young collaborators however I found myself looking at the apparent paradox from a different angle. We reckoned that the amount of free carbohydrate in any form which was available at any one moment in the organism of a small animal such as a mouse would by itself only suffice to meet the needs of its rapid oxidative metabolism for say half an hour or so. Normally the balance was being constantly maintained

by the catabolism of cell proteins with a resulting formation of nitrogenous excreta and glucose which would provide for a long maintenance of the blood sugar even in the absence of food

We thought therefore that the paradoxical effect of insulin could be accounted for if we could suppose that apart from an initial promotion of the synthesis of blood sugar into cell glycogen it would also effectively prevent the production of new glucose from protein. The oxidative metabolism would then be essentially concentrated on the small quantity of carbohydrate available as such at the time of injection and the prevention of new formation from protein would under such conditions become the central feature of the insulin effect and lead to an early collapse and death from carbohydrate exhaustion. The only experimental contribution which we were able to make was to show that in the eviscerated preparation with blood sugar maintained by artificial infusion all the extra sugar disappearing from the blood under insulin could be accounted for by glycogen deposition in the muscles and by oxidation. We had no means available for testing our supposition that insulin would also stop or restrict the new formation of sugar from protein in the liver. I must leave it to our colleagues who have just given us such convincing evidence of the promotion by insulin of synthesis of proteins from amino acids to judge whether the prevention of protein breakdown which we envisaged would not be another aspect of the synthetic action which they have demonstrated.

My only excuse for speaking is that I have had the impression — very probably a mistaken one — that some of the results under discussion earlier today have appeared once again to be producing paradoxical problems of the carbohydrate balance sheet and I have wondered whether these may again have involved a neglect of the factor of the suppression of the new formation of carbohydrate from protein which I still suspect of being perhaps the most important of the actions of insulin. I can only thank you all for tolerating what may be an irrelevant intervention, and for humouring an old man's anecdotalage!

DICKENS There is no need for me to say what a pleasure it was to hear such a clear and vigorous account from Sir Henry who has put the whole thing in proportion by his remarks. We are very glad that he had this accident which meant his entry into this work at that time.

CORI I think of course Sir Henry that new formation of carbohydrate is a process which still exists. The debate was really whether this new formation is from protein only or whether fatty acids are involved in this process as well. During the period which I mentioned earlier when diabetes was explained by over-production then this could only be done by assuming that the fatty acids were giving a net gain in carbohydrates but since we know something about intermediary metabolism of fatty

acids and proteins the present idea is that fatty acids are not important contributors of carbohydrate although labelled fatty acids through a common carbon pool may seemingly show that fatty acids go to carbohydrates. So protein is at the present time supposed to be the main source of the formation of new carbohydrate and it is possible that if insulin promotes the synthesis of protein that this means simultaneously a decreased breakdown of protein. In this sense your point is very well taken that this might very well result in a lessening of new formation of carbohydrate simply by shifting the equilibrium from protein breakdown to one of protein synthesis.

DALE: Do you not think that possibility does come into some of the problems that we were discussing earlier today?

CORI: Yes I should think so very much especially even in diabetes where protein catabolism is increased this action of insulin may well play an important role.





PART VI

BIO-ASSAY OF INSULIN

*Chairman* PROFESSOR T RUSSELL FRASER

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# SUGGESTED IMPORTANCE OF ADIPOSE TISSUE AS A SITE OF INSULIN ACTION AND AS A MAJOR SITE OF METABOLIC INTERRELATIONS BETWEEN CARBOHYDRATES AND FATS\*

ALBERT E. RENOLD   ALBERT I. WINEGRAD   BERNARD JEANRENAUD  
AND DONALD B. MARTIN

*The Baker Clinic Research Laboratory and the Departments of Medicine  
Harvard Medical School and the Peter Bent Brigham Hospital Boston  
Massachusetts*

When considering the metabolic interrelations between carbohydrates and fats it is customary to be primarily concerned with the liver. I should like to review observations which suggest that our concern should be equally devoted to adipose tissue—a tissue highly specialized for the synthesis of fatty acids from glucose as well as from other precursors. With relation to glucose the significance of this problem may perhaps be underlined by recalling that, as first definitely pointed out by Stetten and Boyer, the storage of glucose carbon as fatty acids easily equals and frequently exceeds its storage as glycogen.

Although most of the observations to be reported will concern the effects of insulin upon adipose tissue, I should like to make it clear at the outset that they will not really bear upon the *mechanism* of insulin action. In this tissue we are as yet at the stage of describing *what* insulin does rather than *how* it does it.

The studies to be presented as well as our general interest in adipose tissue and its hormonal control have been early and lastingly influenced by the extensive work carried out in the laboratories of Wertheimer and Shapiro<sup>17, 18, 20</sup> in Jerusalem, Hausberger in Philadelphia<sup>8, 13</sup> and Favarger in Geneva.<sup>5, 6</sup> Wertheimer's classic review published in 1948<sup>20</sup> emphasized the observation that glycogen accumulates in the cytoplasmic rim of adipose tissue cells under certain rather specific circumstances among which the administration of insulin to diabetic rats was prominent. When first

\* Supported in part by the Adipose Tissue Foundation, New York, and the National Institutes of Health, United States Public Health Service, Bethesda, Maryland (Contract No. A-640).

concerned with this tissue in 1949<sup>15</sup> we observed (Fig. 1) insulin was injected into the groin fat pad of six diabetics, being injected as control on the other side) glycogen was greater in the insulin-injected pad suggesting a direct effect of insulin upon this tissue. The glycogen levels shown become quite considerable when related to fat-free tissue.

(PZI 5 units i to left groin fat equal volume saline into right groin fat. Glycogen measured 20 hours later)

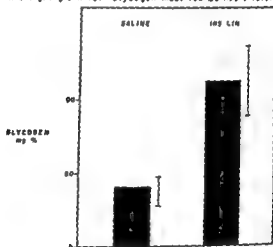


FIG. 1. Local action of insulin in six diabetic rats. Data from Renold et al.<sup>15</sup>

Subsequently the existence of a direct effect of insulin upon adipose tissue suggested here was established *in vitro* by Krahl<sup>16</sup> by Haugaard and Marsh<sup>7</sup> using glucose uptake and oxygen consumption respectively as parameters of insulin effectiveness. The insulin effect upon sliced brown adipose tissue has also, as pointed out yesterday by Chan, but this tissue of almost glassy appearance should probably not be considered as representative adipose tissue in general.

Having paid prolonged and respectful attention to the liver in the intervening years we have recently renewed our interest in adipose tissue using the epididymal fat pad of the male rat as tissue preparation carefully avoiding tearing, slicing, chilling or any unnecessary handling in the process of securing it. This tissue is really

better termed a fat sheet than a fat pad since it pending medium and in the appropriate size rat is  $\frac{1}{2}$  to at most 1 millimetre thick in any one point out to those justly critical of the unhomogeneity existing in isolated tissues that in this instance glycolysis *in vitro* may be shown to proceed rather homogeneously.

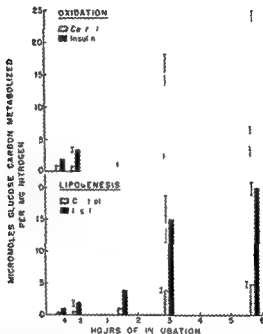


FIG. 1. Effects of insulin added *in vitro* on oxidation of glucose- $U^{14}C$  to  $CO_2$  and on incorporation of glucose carbon into lipid by rat adipose tissue after incubation periods varying from 15 minutes to 6 hours. Incubation carried out in Krebs bicarbonate containing 0 mM/L of glucose. Insulin concentration when present 0.2 unit per ml.

out (unpublished observations of Blacklow and Martin). The tissue is symmetrical and furthermore may be quite conveniently subdivided into as many as three pieces for each pad, a total of six pieces for each animal. Early results concerning insulin effects obtained under these conditions revealed them to be of unexpected magnitude and the decision was made to engage in a rather long-term

programme of re-evaluation of the effectiveness of insulin and other hormones upon this tissue. Some of the observations gathered since<sup>1, 16, 17</sup> will be summarized here.

Fig. 2 illustrates the effects of insulin added *in vitro* at rather high concentration (0.1 unit/ml) upon the oxidation of glucose carbon (using uniformly labelled glucose) to  $\text{CO}_2$  and its utilization for the synthesis of long-chain fatty acids. It is apparent that the effect of insulin is marked early (15 minutes) and persistent (up to 6 hours) although some diminution of effectiveness during the last 3 hours is suggested. I should like to point out that on the balance sheet these two metabolic fates of glucose carbon (i.e. oxidation and lipogenesis) accounted for approximately two thirds of the glucose disappearing from the medium both with and without added insulin. Also that it is not necessary to use labelled glucose: if adipose tissue is incubated in a Warburg flask without centre well in the presence of glucose the respiratory quotient is initially close to unity and net gas formation or uptake is therefore minimal. If insulin is now tipped in from the side-arm the respiratory quotient increases immediately to between 2 and 3 and net gas formation becomes evident proceeding in linear fashion from within seconds of insulin addition to at least 3 hours later. Observations in this system are presently actively being pursued in collaboration with Dr Eric G. Ball in the Department of Biological Chemistry Harvard Medical School.\*

Insulin effects upon adipose tissue occur at remarkably low concentrations of the hormone as shown in Fig. 3. In this instance six pieces of adipose tissue from one rat were used and a clear-cut insulin effect was observed at the concentration of 10 micro-units per ml (or  $10^{-6}$  units per ml) a quite consistent and uniform finding whatever the index of insulin activity used. Still lower concentrations are frequently but not invariably effective. Parenthetically I might recall that the most generally accepted figure for insulin like activity of normal human plasma obtained in the fasting state is of the order of 100 micro-units per ml.

Although effects of insulin added *in vitro* upon fatty acid synthesis have been described in isolated liver these effects could not be obtained in liver isolated from diabetic animals<sup>18, 19</sup>. It was of interest to find therefore that the direct effect of insulin upon glucose oxida-

\* Some of these results have been published by Ball, E. G., Martin, D. B. and Cooper, O. (1959) *J. biol. Chem.* 234: 774.

tion and lipogenesis from glucose could be clearly demonstrated in adipose tissue obtained from diabetic animals as well as the baseline activity of the diabetic tissue and its insulin response was considerably less than normal (Fig 4 second pair of columns). Fig 4 further demonstrates that when the insulin effect upon lipogenesis was tested in the presence of a variety of substrates specifically here in the presence of labelled glucose or acetate.

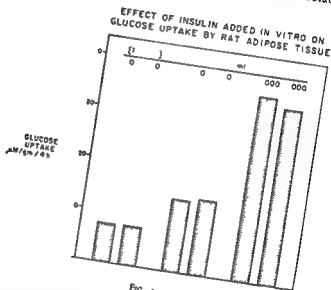


FIG 3

and of labelled acetate with in addition unlabelled glucose) it became clearly apparent that a stimulation of lipogenesis in the presence of insulin required the presence of glucose in the medium. This strongly suggests that accelerated glucose metabolism is a prerequisite for insulin-induced accelerated lipogenesis. This is of course in excellent agreement with present concepts concerning cofactor requirements for the synthesis of fatty acids concepts entirely based upon observations derived from liver preparations (11, 12). In adipose tissue these concepts were further tested by comparing the effects of insulin upon the metabolism of the first and last carbons of glucose. From consideration of the figures summarized in



Table I it is apparent that (as previously shown by Milstein<sup>14</sup> carbon dioxide only) unstimulated adipose tissue metabolizes carbon-1 than carbon-6 to  $\text{CO}_2$  and more carbon-6 than carbon-1 to fatty acids suggesting the likely presence of the phosphoglycerate oxidative pathway in this tissue. In confirmation I have authorized to state that Drs Shaw and Winegrad working in

EFFECTS OF INSULIN ADDED IN VITRO ON LIPOGENESIS  
BY RAT ADIPOSE TISSUE

(mic moles carbon per mg. nitrogen  $\pm$  3 hours)

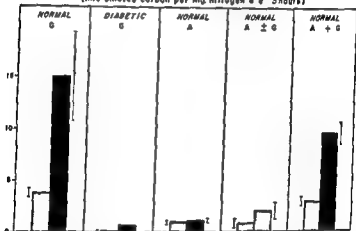


FIG. 4 Each column refers to substrate carbon incorporated into adipose tissue fatty acids during the period of incubation: the black column being the mean activity of the tissues in the presence of insulin (0.1 unit per ml); the shaded column representing the mean of the paired controls. Normal and Diabetic refers to the state of the animals from which the tissues were obtained. The nature of the substrate is indicated by the letters at the top of each pair of columns as follows: G\* glucose- $\text{U}^{14}\text{C}$ , 20 mM/l; A\* acetate- $1\text{-}^{14}\text{C}$ , 60 mM/l; G unlabelled glucose 10 mM/l. Note that in the fourth pair of columns no insulin was added and that the variable examined in this instance is the addition of unlabelled glucose. The bracket adjoining each column indicates the standard error of the mean of the whole group and does not make allowance for the paired nature of the data.

Stadie's laboratory have demonstrated the presence of considerable activity of both glucose-6-phosphate and 6-phosphogluconate dehydrogenases in this tissue. It is also apparent from the table that when insulin is present both metabolic fates of both carbons are activated although not to a similar extent in all instances. I should like to concentrate first on the fatty acid data and point out that the ratio of carbon-1 to carbon-6 incorporated into fatty acids remains the same albeit at a much higher level of activity. The ratio of  $\text{CO}_2$

from carbon-1 to  $\text{CO}_2$  from carbon-6 however is severely shifted towards carbon-1. It is customary to dispose of such discrepancies by pointing out the relative lack of reliability of any calculations based upon  $\text{CO}_2$  data alone (reflecting perhaps variations in pool sizes for various paths from substrate to  $\text{CO}_2$ ) although this reasoning

TABLE I

EFFECTS OF INSULIN ADDED IN VITRO ON THE METABOLISM OF GLUCOSE-1- $^{14}\text{C}$  BY RAT ADIPOSE TISSUE\*

Number of experiments	Insulin	Oxidation $\text{CO}_2$		Incorporation into fatty acids	
		carbon-1	carbon-6	carbon-1	carbon-6
6	0	0.92	0.24	0.19	0.38
6	+	6.36	0.33	1.06	6.31

\* All values expressed as micro moles of glucose carbon-1 or carbon-6 per mg tissue nitrogen. Incubation carried out for 3 hours in Krebs bicarbonate buffer containing 20 millimoles per litre of glucose. Insulin concentration when present 0.1 unit per ml.

does not appear entirely convincing in this instance. Be that as it may however the more standard and rather generally accepted mode of calculating relative activities of the glycolytic and the phosphogluconate-oxidative pathways (based on fatty acid data) demonstrates a completely similar distribution of glucose-6-phosphate metabolism between the two pathways with and without insulin (Table II). We attach importance to this mainly because it clearly suggests that the major action of insulin upon this tissue becomes effective before glucose-6-phosphate leaving increased transport and/or phosphorylation as the only known determinants. Which we could not say.

Parenthetically I should like to add that we have utilized the thus observed highly active  $\text{CO}_2$  formation from glucose-1- $^{14}\text{C}$  as a likely and in our hands the most appropriate indicator of insulin-like activity for bio-assay purposes<sup>14</sup>. The sensitivity of this bio-assay system is 10 micro units per ml ( $13 \pm 4$  per cent of control value 4 being the standard error for twenty observations). This insulin sensitivity is not altered by adrenalectomy or by cortisone overdosage<sup>9</sup> a finding in agreement with the observations of Levine in the eviscerated dog discussed elsewhere in this symposium. Further-

more the depressed lipogenesis from pyruvate exhibited by adipose tissue from alloxan-diabetic rats is not returned toward normal by superimposed adrenalectomy<sup>3</sup> in marked contrast to hepatic lipogenesis from pyruvate under these conditions<sup>4</sup>. This discrepancy could be established even in the same group of animals as illustrated in Fig. 5 and is presently interpreted as suggesting that the adrenal

TABLE II

EFFECT OF INSULIN UPON THE METABOLISM OF CARBON-6 OF GLUCOSE TO FATTY ACIDS  
BY WAY OF EMBDEN MEYERHOF OR ALTERNATE PATHWAYS\*  
(Calculation discussed in Reference 21)

Animal no	Total	% in Embden Meyerhof pathway	Via alternate pathway	% in alternate pathway (per cent of total)
No insulin added				
116	0.37	0.24	0.13	35
117	0.66	0.4	0.26	64
118	0.62	0.35	0.27	66
12	0.41	0.15	0.26	63
13	0.25	0.14	0.11	44
124	0.23	0.10	0.13	57
Mean				55
1 unit/ml added to all flasks				
119	6.38	2.64	3.74	59
120	6.34	2.91	3.43	54
121	2.91	1.1	1.79	62
125	9.59	5.00	4.59	48
126	4.19	2.39	1.80	43
127	3.48	4.8	3.66	43
Mean				52

\* See footnote Table I

cortical effect upon hepatic lipogenesis may well be secondary to other hepatic metabolic events induced by glucocorticoid administration or withdrawal.

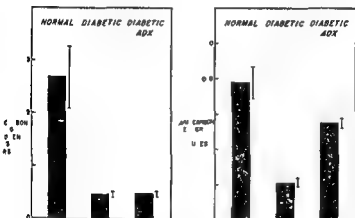
I should like to devote the last minutes of this presentation to two as yet somewhat unrelated observations which I would use mostly to emphasize the probably multiple nature of the regulatory effects exerted upon adipose tissue effects which we are only just beginning to analyse. I am fortunate in being able to present work from both present and past collaborators some very recently obtained. First

Albert Winegrad now working in the laboratories of Dr Stadie. Dr Lukens has recently expanded observations begun jointly on the effects of bovine growth hormone on adipose tissue. We had previously found that growth hormone added stimulates glucose uptake stimulates  $\text{CO}_2$  production but

## LIPOGENESIS FROM PYRUVATE $2\text{ C}^{14}$

BY RAT ADIPOSE TISSUE

BY RAT LIVER SLICES



Adipose tissue and liver slices were obtained from the same animals. Note that only the values for the basal rate should be compared between tissues in the controls of the incubations with regard to time as well as the reference measurement differed. The bracketed group each column indicates the standard deviation of the mean for each group (unpublished data of Jeanrenaud and Renold).

to stimulate lipogenesis. When as shown in Fig. 6 the oxidation of carbon-1 and carbon-6 was measured in the same tissue at early intervals for 4 hours unstimulated tissue persistently produced more  $\text{CO}_2$  from carbon-1 than from carbon-6 and the addition of insulin at the end of the first hour resulted in a much greater increase of  $\text{CO}_2$  production from carbon-1 than from carbon-6 as expected from observations already discussed (Table I). When growth hormone however was added after the first hour apparently selective stimulation of the oxidation of carbon-6 resulted. A possible explanation of this rather unexpected finding

would suggest a specific stimulation of the glucuronic acid pathway \* It is necessary to point out the high concentration of growth hormone required to exert this effect upon this tissue

Secondly Drs Peter Bally and George Cahill in our laboratory have studied the effects of glucose upon the incorporation of palmitate-1- $^{14}$ C into adipose tissue triglyceride and upon its oxida

EFFECT OF INSULIN (0.1 U/ml) & BOVINE GROWTH HORMONE (1 mg/ml) ON THE OXIDATION OF CARBON 1 & 6 OF GLUCOSE BY ADIPOSE TISSUE OF NORMAL FED RATS

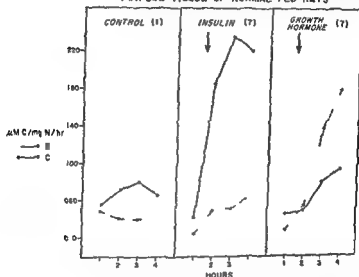


Fig. 6 Each point represents the hourly rate of  $^{14}\text{CO}_2$  production. In the case of insulin and growth hormone the addition of either hormone immediately followed the  $\text{CO}_2$  collection at the end of the first hour. Incubation was carried out in Krebs phosphate buffer (observations of Winegrad, Shaw, Lukens and Stadie)

tion to  $\text{CO}_2$ . As illustrated in Fig. 7 the presence of glucose at quite low concentrations increased fatty acid storage but decreased fatty acid oxidation \*\*. Also Gordon recently reported a direct insulin effect upon fatty acid release by adipose tissue in the presence of glucose

\* These studies have since been published by Winegrad, A. J., Shaw, W., Stadie, W. C., Lukens, F. D., and Renold, A. F. (1960) *J. Biol. Chem.* 234: 192.

\*\* These studies are now in process of publication by Bally, P. R., Cahill, G. F., Jr., Leibel, R. L., and Renold, A. E. (1960) *J. Biol. Chem.*

I should like to begin the summary by pointing out that according to the schema prevailing 10 or 15 years ago glucose (with regard to its relations to fat metabolism) was primarily transported as glucose to the liver there transformed into fatty acids transported as fatty acids or triglycerides to adipose tissue for storage recalled

EFFECT OF GLUCOSE UPON INCORPORATION OF  
PALMITATE- $C^{14}$  INTO TISSUE LIPIDS AND  
UPON ITS OXIDATION TO  $CO_2$   
BY RAT ADIPOSE TISSUE

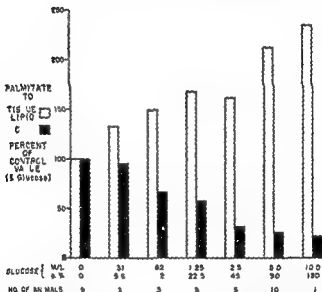


FIG. 7. In what was carried out in Krebs' bicarbonate buffer with 1 per cent dialysed human albumin and 2 mEq/l. palmitic- $C^{14}$ . Note that the values are expressed as per cent of the control value obtained in the absence of glucose (data of Bally, C. and LeBeauf and Renold).

when needed to the liver and there transformed into ketone bodies and thus finally made available for general consumption by tissues such as muscle. In accord with newer knowledge this schema has been largely replaced by direct fatty acid synthesis from glucose in adipose tissue itself and direct utilization of fatty acids released from adipose tissue not only by the liver but also (and to a major extent) by tissues such as muscle, in particular heart muscle. This being

should we not really expect a highly sensitive regulatory system for fatty acid synthesis fatty acid release by this tissue? We believe that to be the case and feel that we have found as evidence of a direct action of at least one hormone adipose tissue an action exerted upon glucose synthesis glucose oxidation and fatty acid magnitude of the effect of insulin upon lipogenesis<sup>1</sup> and the low concentration of insulin required may well be the main site of insulin action upon<sup>1</sup> data we have gathered to date are for the time being with a primary insulin effect upon this tissue at an glucose utilization specifically between extracellular intracellular glucose-6-phosphate while not of co other sites of action on parameters not measured As made no distinction between an effect of insulin transport or phosphorylation

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# INSULIN IN SERUM PROTEIN FRACTIONS

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Measurements of the glucose uptake of the isolated rat diaphragm have now been shown by many workers to be the basis of a sensitive mode of insulin assay *in vitro*. However when the method is applied to blood its reliability may be seriously affected by the presence of other substances in blood. This is especially the case where the blood serum of diabetic man or animals is concerned. Thus Randle and Young<sup>15</sup> and Bornstein<sup>7</sup> have described lipoprotein type inhibitors of glucose uptake by the diaphragm in diabetic men or animals. Field and Stetten<sup>9</sup> claim to have detected an  $\alpha$  globulin inhibitor of insulin action in the serum of ketosed human diabetics and more recently Vallance-Owen *et al*<sup>18</sup> have described an insulin inhibitor present in the serum albumin of patients with uncontrolled diabetes. According to Groen<sup>10</sup> and his co-workers adrenaline too may inhibit the action of insulin even when present in concentrations which are stated to be physiological.

There are moreover many substances which may by themselves simulate the action of insulin thus certain small molecule substances such as salicylate or dinitrophenol are now known to increase glucose uptake by the diaphragm and serum albumin has been reported to do the same.<sup>12, 14</sup>

A further difficulty relates to the form in which circulating insulin is present in blood. From the studies of Berson *et al*<sup>4</sup> it seems likely that some of the insulin present in the serum of insulin-treated diabetics may be bound to globulin by what is apparently antibody and this antibody bound insulin may not be necessarily available for action on the diaphragm. It is also possible that not all the insulin in normal serum is biologically available when undiluted serum is employed and tested on the diaphragm.

These considerations led to an attempt to fractionate serum protein for insulin activity. The method which we have found most

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valuable has been zone electrophoresis on treated cellulose columns. In general 5 ml of serum have been electrophoresed for 40 hours at 1000 volts in a phosphate-borate buffer pH 8.4. After electrophoresis the separated proteins were eluted from the column and the protein concentration of 5 ml portions of the eluate determined by measuring the optical D at 280 m $\mu$  in the Beckman spectrophotometer. In order to locate insulin in the serum protein fraction a trace labelled  $^{131}\text{I}$  insulin has been prepared and electrophoresed in the presence of serum. The results show that a peak of radioactivity appears migrating somewhat slower than the albumin fraction. This confirms the observations of several other groups of workers that free insulin migrates on starch blocks or paper with a mobility slightly less than that of serum albumin at or about this pH.

In these experiments especial attention was paid to the possibility that the insulin itself might have been damaged by iodination so that its electrophoretic mobility was altered since Berson<sup>6</sup> has reported that this type of damage may take place using radio-iodine. The preparation obtained however had a similar mobility on electrophoresis to that of un-iodinated insulin and behaved similarly when subjected to paper chromatography in a butanol/acetic system.

The next series of experiments were concerned with the effect on the glucose uptake of the diaphragm of serum fractions obtained by electrophoresing serum mixed with excess unlabelled insulin. In this experiment fractions were dialysed against distilled water, freeze-dried and the dry protein residue dissolved in buffered glucose before testing on the diaphragm. A maximal effect is obtained with the slower moving albumin fractions with some smaller effects on the  $\beta$  and  $\gamma$  globulins (see Fig. 1).

When normal serum was electrophoresed without additions the greatest effect was found again in the slower-moving albumin fractions with somewhat lesser activity appearing in the  $\beta$  and  $\gamma$  globulins. These results have been reported in full elsewhere.<sup>16,17</sup>

Broadly similar results have been obtained by testing fractions made by electrophoresing the serum of diabetic patients treated with insulin, stimulation being obtained in the slower- but not the faster-moving albumin fractions and also in the  $\beta$  and  $\gamma$  globulins.

In recent publications both Beigelman<sup>3</sup> and Bolinger *et al*<sup>6</sup> have independently reported that insulin activity is present in the  $\beta$ - and

$\gamma$ -globulin fractions obtained by the electrophoresis of normal human serum though some doubt seems to exist as to whether or not albumin may also exhibit this on occasions

In a general sense these experiments agree with those reported above although the effects we have noted in the albumin zone appear to be somewhat greater than theirs

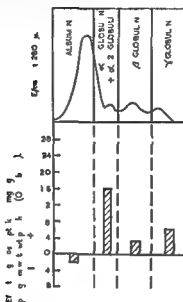


FIG. 1. The effect of insulin on the uptake of the solid red phosphorus. Experiments in which insulin was added to the fractions before electrophoresis.

Since labelled and unlabelled insulin added to serum migrate in the slower moving albumin zone it seems probable that the activity found in this region when serum to which additions have been made is electrophoresed is due in fact to free insulin present in the serum. However some doubt as to the exact nature of the stimulating effect due to the  $\beta$  and  $\gamma$  globulin remained and it was decided therefore to see if this effect was neutralizable by insulin antiserum preparations. Since human insulin was not available it was decided to prepare antiserum against ox insulin and to test this against ox-

serum protein fractions Antisera to ox insulin were prepared in guinea-pigs by the method of Moloney and Coval<sup>1</sup> using an emulsion of insulin in oil Such preparations will completely neutralize the effect of insulin in stimulating the glucose uptake of the isolated rat diaphragm Control preparations made by injecting oil alone into guinea-pigs did not neutralize insulin Neutralization of the effects of the insulin on the rat diaphragm have also recently been reported by Birkenshaw *et al*<sup>8</sup>

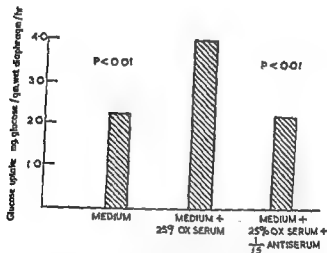


FIG. 2 The effect of antiserum to ox insulin prepared in guinea pigs on the stimulation by ox serum of the glucose uptake of the isolated rat diaphragm

It was first decided to attempt to neutralize the effects of whole ox serum by the antiserum preparation In Fig 2 are shown the results of such an experiment

The ox serum was tested in 25 per cent dilution with an antiserum concentration of 1 in 15 Complete neutralization of the stimulating effect of the serum has been obtained These results have been obtained with other specimens of ox serum

The results of electrophoresing ox serum in parallel with the effects of various fractions on the glucose uptake of the isolated rat diaphragm are shown in Fig 3

For purposes of investigating effects on glucose uptake broad divisions of fractions have been made into albumin and post-albumin one and  $\beta$  and  $\gamma$  globulins It will be seen that while the whole

albumin fraction has no effect on glucose uptake there is a small effect in the post-albumin zone a greater effect in the  $\beta$  globulins and the greatest effect in the  $\gamma$  globulins. These effects appear to be neutralizable by anti-sera as can be seen in Fig 4 using another sample of antiserum

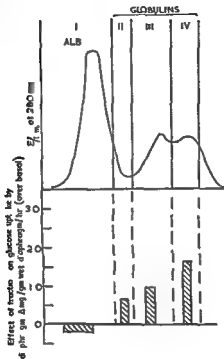


FIG 3 The effect of fractions separated by the zone electrophoresis of normal ox serum on the glucose uptake of the isolated adipose gm.

In the left half of the figure are shown the neutralizing effects of antiserum on the  $\beta$  globulins and post albumin fractions and on the right are shown the effects on the  $\gamma$  globulins. From these results therefore it seems that all the effects of ox-serum fractions on glucose uptake are neutralizable by ox insulin antisera. Similar results have been obtained with the  $\gamma$  globulins prepared by electrophoresis from several other ox-serum samples.

Complete neutralization of the effects of the whole serum of an insulin-treated diabetic patient have also been obtained as well as neutralization of the  $\gamma$  globulin of the same patient. It seems therefore that if the antiserum test is a specific one these  $\gamma$  globulin effects are due in fact to insulin.

I would like now to return to the effects of serum albumin itself on glucose uptake. Several workers have in the past reported that serum albumin enhances the uptake of glucose by the isolated rat

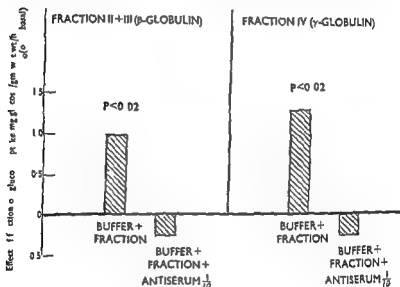


FIG. 4. The neutralization of the insulin effect of ox serum protein fractions by antiserum to ox insulin prepared in guinea pigs.

diaphragm and it has been suggested by Randle<sup>14</sup> that this effect might be due to a contamination of the albumin by insulin. Column electrophoretic experiments support this conclusion. In these experiments the whole albumin fraction of ox serum and the faster-moving fractions of human albumin do not enhance glucose uptake; indeed there is if anything a slight inhibition. Albumin from several species however made by the precipitation method of Korner and Debro<sup>11</sup> does enhance uptake of the diaphragm — the effect being greatest in the ox.

In Fig. 5 are shown the effects of human albumin prepared by this method on glucose uptake compared with the effects of albumin derived from human serum to which a small quantity of insulin had been added before precipitation. In this case the albumin preparations were precipitated with trichloroacetic acid, extracted with alcohol, dialysed against distilled water, lyophilized and after making up in buffered glucose were tested on the diaphragm. A small effect is seen with the sample made from serum without

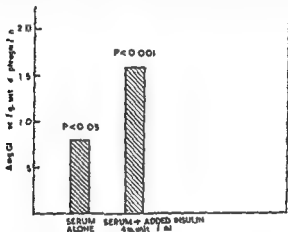


Fig. 5. The effect of albumin prepared by the fractional precipitation of proteins from normal serum on the glucose uptake of the isolated diaphragm.

additions and a very much larger effect with the sample to which insulin had been added. These results indicate that insulin may be co-precipitated with serum albumin under these conditions. Albumin itself appears to be without insulin activity and indeed the results of the work of Vallance-Owen *et al*<sup>18</sup> indicates that an antagonist may be present in this fraction.

Before drawing some general conclusions mention should be made of more recent work on the serum of insulin treated diabetics. It was noted in some experiments that the effect of the  $\gamma$  globulin fractions obtained by electrophoresis of serum of these individuals was considerably greater than normal even though the whole serum itself had no statistically significant effect on glucose uptake.

These results led to an attempt to extract insulin from this type of serum by an acid-alcohol method. After dialysis against water freeze-dried extracts were made up in buffered glucose and tested on the diaphragm. The results of such an experiment showing a large  $\gamma$ -globulin effect are shown in Fig. 6. In this case while the unfractionated serum is without effect on glucose uptake a larger effect is obtained with the extract. These results confirm the work of *Barclay and Bornstein* who have shown by a similar extraction technique that insulin may be present in the serum of such individuals though its biological activity may in some way be masked.

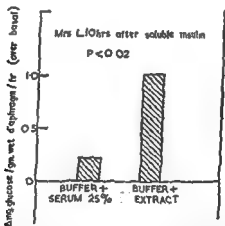


FIG. 6 The effect of serum and crude acid-ethanol extracts of serum of an insulin treated diabetic patient on the glucose uptake of the isolated rat diaphragm.

To summarize it seems that in whole serum all the effects in stimulating glucose uptake are due to insulin. Likewise the stimulating activity of plasma protein fractions seems also to be due to insulin if insulin antiserum represents a specific insulin antagonist. We have found no evidence that albumin itself free from insulin stimulates the uptake of glucose by the diaphragm.

Electrophoresis of human serum has revealed stimulatory effects on glucose uptake in two zones namely the slower-moving albumin zone and the  $\beta$  and  $\gamma$  globulins. Since free insulin labelled or unlabelled added to blood appears to migrate in the slower-moving albumin zone the effect observed in this zone in normal serum after

electrophoresis would seem to represent free circulating plasma insulin

Studies with antisera also suggest that the effects noted in the  $\beta$  and  $\gamma$  globulins are due to insulin. If this is so then this insulin may be associated in some way with the globulins. Berson<sup>8</sup> has suggested that globulin-bound insulin is present in the serum of insulin-treated diabetics and it is not impossible that similar association of insulin with  $\beta$  and  $\gamma$  globulins takes place even in normal serum. The idea that insulin may in some way be associated with serum proteins is not entirely new. Using ion exchange columns Antoniadou et al.<sup>1</sup> has also reported that insulin added to blood may behave differently from the circulating hormone and it has been suggested that this may be due to the association of insulin with serum proteins.

Finally the possibility that some of the blood insulin of treated diabetics may be bound to  $\gamma$  globulins and therefore biologically unavailable may be a clinically important one and this deserves full investigation.

#### ACKNOWLEDGMENTS

Professor F. G. Young has my best thanks for help and encouragement throughout the investigations.

I would also like to record my thanks to Dr I. B. Cole, Addenbrookes Hospital, Cambridge, for permission to use data on his patients and to the British Diabetic Association and of the Elmore Research Fund for grants towards my expenses.

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# BIO-ASSAY OF SERUM INSULIN-LIKE FACTORS\*

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There has been abundant evidence that alterations in adipose-tissue metabolism are induced by insulin<sup>4,5,8</sup>. More recently it has been noted that increments of glucose uptake by adipose tissue are responsive to such small concentrations of insulin that an insulin bio assay sensitive to 10 micro-units/ml may be feasible with this system<sup>2,6</sup>. For the past year this technique of bio-assay for insulin-like activity utilizing glucose uptake by wistar rat epididymal adipose tissue has been studied at the University of Southern California Medical School. This method has proved highly sensitive and quite simple but does not appear as yet to be very precise.

## METHODS

Fed male Wistar rats were stunned by a blow to the head and quickly killed by decapitation. Segments of epididymal adipose tissue were rapidly excised, quickly weighed in a torsion balance and immediately added to Krebs bicarbonate buffer containing glucose. Human sera and concentrations of insulin were added to the buffer and glucose substrate. 0.1 per cent albumin was incorporated with the controls and the insulin determinations. Incubation proceeded for 2-4 hours in a Dubnoff metabolic shaker under 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub> at 36.5°C. The usual total glucose was 0.3-0.5 mg in 0.5 ml volume of buffer per beaker. Residual glucose was measured by the Somogyi-Nelson method<sup>7</sup> and glucose uptake expressed as mg glucose/g adipose tissue.

## RESULTS

A number of variables including weight of the animal, initial glucose concentration, duration of incubation, weight of adipose tissue, state of nutrition and protein concentration are being studied. At present the weight of the animal appears to be the most impor-

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tant factor. Higher glucose uptakes and an improved response to the insulin were associated with employment of adipose tissue from lower weight animals. The correlation coefficient ( $r$ ) for animals in the 100-180 g weight range was 0.54 ( $P < 0.001$ ). Conversely the  $r$  value was 0.22 ( $P > 0.4$ ) for rats weighing more than 350 g (Fig. 1). Suggestive loss of insulin sensitivity also appears to be associated with increased weight of tissue and decreased duration of incubation. Preliminary evidence indicates that alterations of initial

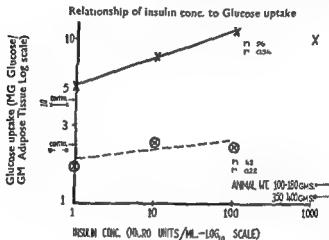


FIG. 1. Relationship of glucose uptake (mg glucose/g adipose tissue) expressed as  $\log_{10}$  to insulin concentrations  $\log_{10}$  values (all values on graph represented as anti  $\log$ ) of 1, 10, 100 and 1000 micro-units/ml. Control uptake also represented. Relationship of increment of glucose uptake to insulin concentration evident in 1-100 micro-units/ml range. Correlation coefficient ( $r$ ) calculated for this dose range.

glucose concentration within the range 2.0-4.0 mg do not materially affect bio-assay sensitivity. There is some suggestion of slight insulin-like activity associated with albumin concentrations of 1.0 per cent or greater, but the evidence is not conclusive. At present 40-60 mg segments of adipose tissue from rats weighing 100-180 g are incubated for 2-3 hours in Krebs bicarbonate buffer containing 2-3 mg of glucose per beaker.

Clinical studies have been performed on normal and diabetic coma sera. There is considerable individual variation of serum (diluted 1/4 or 1/10) insulin-like activity in fifteen normal subjects studied. To some extent this may reflect variability.

of the method but also indicates wide fluctuations of normal serum insulin-like activity. Preliminary evidence suggests that 2-hour post-prandial serum insulin-like activity is greater than that of corresponding fasting sera.

Serial serum insulin-like activity determinations have been performed on eight diabetic coma patients.<sup>2</sup> The initial pre-treatment coma specimens demonstrated usually little or no insulin-like activity. Rapid increment of serum insulin-like activity accompanied exogenous insulin therapy and did not appear to be associated with any alteration of the patients' clinical status. Certainly there was no very apparent relationship between the increase of serum insulin-like activity and change in blood glucose levels or CO<sub>2</sub>-combining power.

Investigation of insulin-like activity in various discrete serum protein fractions prepared by the method of continuous flow paper electrophoresis was performed in this bio-assay system. The technique of continuous flow paper electrophoresis has been described.<sup>1</sup> Serum albumin,  $\gamma$  globulin and  $\alpha_2$ -globulin definitely demonstrated no insulin-like activity.  $\beta$  globulin and adjacent fractions, particularly the fraction migrating between  $\beta$  and  $\gamma$  globulins, were associated with increased glucose uptakes. The fraction which migrated between  $\beta$  and  $\gamma$  globulins possessed definite and significant insulin-like activity.

### CONCLUSIONS

The method of bio-assay for insulin-like substances utilizing glucose uptake by rat epididymal adipose tissue is sensitive to 10 micro-units of insulin/ml and sensitivity on occasion has been noted to as little as 1 micro-unit of insulin/ml. This technique is relatively simple but the method does not possess a very high degree of precision which may be an inherent characteristic of this being a protein hormone bio-assay. Considerable refinement of this method is possible as various modifying factors are corrected. The animal weight appears to be a factor of primary importance, the bio-assay sensitivity being inversely proportional to the weight of the rat.

Clinical studies indicate considerable variation of insulin-like activity in normal sera. Pre-treatment diabetic coma sera possess little or no insulin-like activity. Marked increase of activity is

associated with exogenous insulin administration but does not appear to correspond to clinical response

Studies by this method of serum protein fractions obtained by continuous flow paper electrophoresis indicate predominant insulin like activity in the  $\beta$ -globulin area. These observations are in accord with the results obtained by testing similar fractions in a crude insulin-assay system utilizing blood glucose decrement of intact anaesthetized mice<sup>1</sup>

It is of the utmost importance to recall that this method with other techniques of bio-assay does not specifically measure insulin but only expresses a non-specific change namely glucose disappearance. It is probably justified to make the assumption that this reflects uptake of glucose by the adipose tissue and one may even be permitted to equate glucose uptake with insulin-like activity. However to attribute such an effect solely to insulin would be an error and the term insulin-assay therefore is a misnomer. Multiple insulin-like factors some possibly unrelated to insulin may be operative in a fashion similar perhaps to the several anti-insulin or insulin-inhibitory factors which have been characterized<sup>2</sup>

### SUMMARY

A method of bio-assay for insulin-like activity utilizing glucose uptake by rat epididymal adipose tissue has been developed which is sensitive to as little as 10 micro-units of insulin/ml. This technique has been applied to clinical studies and suggests wide variability of normal serum insulin-like activity. Pre-treatment diabetic sera demonstrate little or no insulin-like activity. Serum protein fractionation studies indicate that insulin-like activity is associated with  $\beta$  globulin or adjacent globulins.

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## DISCUSSION

WRIGHT I find it rather hard after these three papers ground which I can use as a basis for opening this heard about the use of two *in vitro* systems and they in different ways and with different ends in view. I want to be thoroughly selfish and talk about what interests applicable I think to the remarks made by the last two.

They have referred to biologically active substances which in effect in the epididymal pad and on the rat. Dr Taylor has made a positive attempt to identify the material. When we are considering substances or extracts which might contain insulin we must consider it might be used to establish the identity of that substance.

It is unlikely that purely chemical or physical methods to the identification of small amounts of insulin mixed or relatively large quantities of contaminating proteins in body as plasma or their extracts. The biological properties of insulin be used for its detection and assay and it has to be shown that logical responses produced by suspected material *in vitro* and identical with those of insulin.

Many substances other than insulin are known to exert hypoglycaemic effects in animals amongst these are the sulphonyl ureas, phenethyl biguanide. Others which may or may not exert hypoglycaemic effects in animals will stimulate glucose consumption. Isolated rat diaphragm, synthalin, phenethyl biguanide, arsenite etc. fall into this category. It may be possible to distinguish other biologically active substances by observing the pattern of biological response produced: thus phenethyl biguanide causes a 2-fold increase in lactic acid production in the rat diaphragm which is not the case of insulin. By such methods one may be able to distinguish insulin as the cause of biological activity in an extract but more positive evidence is necessary to identify insulin as the active component. Activity is destroyed after treatment with cysteine, pepsin, insulin alkali if it is concentrated by extraction of the tissue by a method designed to extract insulin or if it moves under electrophoresis at a rate similar to that of insulin it is likely that the active component is in fact insulin. Now another aid can be used which is more specific in its action involves a simpler technique.

Moloney and Cosal (*Biochem J* 1955 59 179) have shown that insulin injected repeatedly into guinea pigs serum from such an animal when mixed before injection with insulin will protect mice from convulsive action of the hormone. I have found that if serum (1-5 cent v/v) from guinea pigs sensitized in the same way to purified bovine

insulin is added to the incubation medium at the same time as insulin (approx 1 mull-unit/ml) it will abolish the stimulant effect of the hormone upon glucose consumption by the isolated rat diaphragm. Normal guinea-pig serum has no such action. Serum from animals sensitized to bovine insulin will also abolish the stimulant actions of similar concentrations of pig, sheep and human insulin (Fig 1). It has no effect in similar concentration upon the stimulant actions of synthalin (5 mg/100 ml) or phenethyl biguanide (50 mg/100 ml). Moloney and Cova<sup>1</sup> concluded that such serum contains antibodies and it appears that they are specific though not species specific for insulin. I have no evidence which would contradict this.

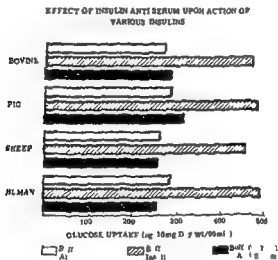


FIG 1

Serum from guinea-pigs sensitized to bovine insulin has been used to aid in the identification of insulin in plasma and its extracts. It is known that plasma and some of its protein fractions exert a hypoglycaemic effect in ADH and ADHA rats and as shown by Dr Beigelman and Dr Taylor that they stimulate the glucose consumption of the isolated rat diaphragm and of the epididymal fat pad. In the case of bovine plasma and one of its protein fractions (Armour Fraction V) I have made similar observations using the isolated rat diaphragm and have shown that in both cases the stimulant effect is abolished by treatment with cysteine or with the insulin antiserum (Wright *Proceedings of 3rd Congress*

of International Diabetic Federation (in press)) normal has no effect

Recently a man was convicted for the murder of his insulin (Birkshaw *et al* *Brit med J* 2 463) The underlying injection marks found on the dead woman extracted by the normal method used commercially for from the pancreas. These extracts were injected into to have a hypoglycaemic effect equivalent to that of 80 This hypoglycaemic activity was abolished after treatment with insulinase or cysteine and the biological response in guinea pigs was the same as that of an equivalent dose of the rat diaphragm. I found that these extracts stimulated summation and that their activity was reduced or abolished with cysteine or pepsin. I was also able to show that the serum but not normal serum from guinea pigs abolished like activity of the extracts.

These experiments lead me to believe that if the biologic tissue or its extract can be abolished by treatment with insulin sensitized animals then the active material present. However the converse may not necessarily be true where activity is not destroyed it is still possible that insulin may be the agent.

RUSSELL FRASER. The papers this morning have clearly important next step to explore the uses of these bio-assay procedures have shown already how far this procedure has gone. Dr P is of course throwing a new light on the relevance of obesity to I do not know whether he would like later to comment on any. he has got that insulin may influence the release of glucose from adipose tissues in disease or in normal circumstances.

In regard to the insulin in serum fractions it still seems a little to be sure but perhaps some of the sites as with many other are more specific than others.

I have much pleasure in throwing these and other points of discussion.

CHAIRMAN. I should like to comment on Dr Renold's paper. In our view the strong effect of insulin on fat synthesis supports the view that insulin steps up the energetic potential of the cell. In the case of a energy requiring action such as the fatty acid synthesis this must be accompanied by a concomitant increase in oxygen uptake or there would be no possibility of deriving the energy for this fatty synthesis.

The effect of insulin on fatty acid synthesis has of course been served in various tissues including the lactating mammary glands.



fact Dr Folley and his group have used the strong effect on the increased respiratory quotient in that system as an *in vitro* measure of insulin activity. It seems to me a very sensitive method for assaying insulin and it has not been mentioned at all this morning.

An increased oxygen uptake cannot be obtained by increasing glucose concentration and here again we have got an insulin effect which cannot be simulated by glucose concentration. I should say the situation resembles that which Britton Chance has seen in his system where he can get an increased oxygen stimulation by supplying free ADP. As long as

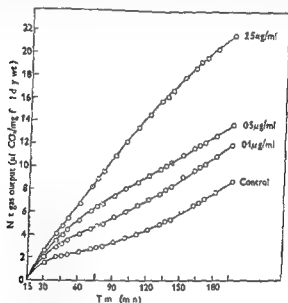


FIG. 2. Manometric *in vitro* assay of insulin based on the measurement of the extra CO<sub>2</sub> output produced by insulin in lactating mammary gland tissue. (From *Selected Scientific Papers from the Istituto Superiore di Sanità* 1956, Part III, p. 440. Present knowledge of the mechanism of the mode of action of insulin by Chan et al.)

ADP is phosphorylated up goes the oxygen which means in other words as long as there is an energy demand on the part of the cell it is supplied by the response of oxygen. As soon as the ADP is fully transformed into ATP the oxygen consumption goes down. I think it is when one sees an increase of oxygen by adding a substrate to the tissue provided of course saturation level is reached it means one of two things either the cell is carrying out mechanical or chemical work or it is cheated into believing that there is a demand for energy requirement by an uncoupling mechanism. You see this with dinitrophenol and so on. The tissues in

which fat synthesis takes place such as the white adipose tissue or the lactating mammary gland tissue provide we believe an ideal system for studying the connection between the respiratory chain and the coupling to the synthetic reactions in the particular case of the brown adipose tissue two synthetic reactions fat synthesis and glycogen synthesis are accelerated by insulin. I was interested to hear that this problem is being studied by Dr Ball we are engaged in similar studies trying to use spectrophotometric methods.

This is the monometric method based on the increase of the respiratory quotient. This system responds to 0.1  $\gamma$  per ml of insulin. I thought it would be interesting for you to see it.

TABLE I

LOG DOSE RESPONSE CURVES FOR THE EFFECT OF INSULIN ON THE INCORPORATION OF  $^{14}$ C GLYCINE INTO DIAPHRAGM PROTEIN IN VITRO

Insulin concentration (mU/ml)	Effect of insulin Mean difference $\pm$ SE of difference (counts per min)	Significance of difference (P)
0.0125	$-1 \pm 9.3$	
0.05	$37 \pm 13.4$	$<0.2$
0.20	$71 \pm 13.4$	$<0.001$
0.80	$99 \pm 10.8$	$<0.001$
3.20	$113 \pm 12.5$	$<0.001$
12.80	$116 \pm 13.4$	$<0.001$

Each value is the mean of twelve determinations.

Incorporation (counts per min) in the absence of insulin =  $158 \pm 4.5$ .

MANCHESTER I would like to mention another insulin bio-assay that Dr P. J. Randle and I have developed. I pointed out in my paper yesterday that insulin at concentrations as low as 0.5 mU/ml (2.5  $\mu$ g/ml) stimulates incorporation of amino acids into protein of isolated rat diaphragm. In Table I is shown a log dose response curve (the combined results of two experiments performed by Dr Randle and myself) for the effect of insulin *in vitro* on the incorporation of  $^{14}$ C glycine into the protein of isolated rat diaphragm. The concentration of insulin was varied in fourfold steps over the range 0.0125–12.80 mU/ml. Increase in the concentration of insulin added consistently increased the magnitude of the stimulation of incorporation produced by insulin over the range of insulin concentrations studied.

Analysis of regression of results of the two experiments contained in Table I is shown in Table II. The two experiments are here treated separately. In both cases regression is highly significant and the deviation from regression very small. The regression is therefore almost certainly linear. Since the slope of the log dose response curve is shallow (the

TABLE II

ANALYSIS OF REGRESSION FOR THE EFFECT OF INSULIN ON THE INCORPORATION OF  $^3\text{H}$  GLYCINE INTO DIAPHRAGM PROTEIN *IN VITRO*

Exp no	Source of variation	Mean square	F	P	Slope $\pm$ S.E. of slope	$\bar{r}$
1	Linear regression	39.647	22.3	<0.001	$4.7 \pm 6.7$	0.39
	Deviations from regression	764	0.43	>0.05		
	Random sampling	1775				
2	Linear regression	20.093	13.9	<0.01	$30.4 \pm 6.3$	0.51
	Deviations from regression	533	0.37	>0.05		
	Random sampling	1446				

maximum increase of incorporation that can be produced by addition of insulin is only of the order of 50 per cent of the basal level — Manchester and Young *Biochem J* 1958 70 353) the degree of precision of the assay ( $\lambda$ ) is not greatly superior to other existing insulin assays.

The insulin activity of two samples of human plasma collected from normal people 2 hours after oral glucose has been measured by this method. The results 2 milli-units/ml and 10 milli-units/ml (Table III) are of the same order as have previously been obtained by Dr Randle (Randle *Ciba Colloquia Endocrinology* 1957 11 115) by the use of the glucose uptake of isolated rat diaphragm as his assay.

TABLE III

MEASUREMENT OF PLASMA INSULIN ACTIVITY BY ITS EFFECT ON THE INCORPORATION OF  $^{14}\text{C}$  GLYCINE INTO DIAPHRAGM PROTEIN *IN VITRO*

Counts per minute in protein Mean $\pm$ S.E. of mean				
Control	Insulin (milli-units/ml) 0.24      1.0		Plasma fourfold dilution	Calculated insulin activity of plasma (milli-units/ml)
Assay 1	$249 \pm 8$	$300 \pm 10$ $325 \pm 7$	$323 \pm 3$	10
Assay 2	$327 \pm 17$	$443 \pm 16$ $489 \pm 28$	$468 \pm 30$	2

Each figure is the mean of eight observations.

Blood samples collected from normal people 2 hours after oral glucose (50 g).

STEWART: I was recently reading some early literature and found that Best and his colleagues many years ago had extracted the blood insulin or an insulin-like substance with the mean activity of 29.3 milli-units/c.c. Even in the diabetic animal there was a level of 16.0 milli-units on average.

We are talking nowadays in terms of micro-units of insulin and I want

to ask Dr Taylor whether he had actually assayed it, he had prepared. If these substances are not insulin, the potent materials.

TAYLOR: In answer to Mr Stewart I would say that any very exact quantitative work yet on acid alcohol, there is no doubt that with the kind of procedure we are down to a level say of 1 mill unit/ml in an extract up and will certainly come through the extraction procedure. I cannot say more about the quantitative nature of this. I do not know why Best got these very high figures. I have no explanation for that.

RENOLD: I wish to thank Professor Chaim for his work on mammary gland which he referred to is of course, although it should be pointed out that the lowest effective concentration of insulin in this tissue is 2000  $\mu$ g/ml as compared to the minimum concentration of 10  $\mu$ g/ml for adipose tissue. Balmain has concluded from these studies that the system was inadequately sensitive for the bio-assay of insulin in serum. With regard to the significance of the observation in mammary gland it may be called that the effectiveness of insulin is limited to lactating rat gland and was not apparent in sheep mammary gland. I have no comments concerning the nature of the energizing effect postulated by Professor Chaim. The effect of insulin consumption in this tissue is surprisingly small and indeed significant even when major acceleration of glucose utilization is demonstrated.

In closing I may perhaps be permitted to say that I was a little disappointed by the limitation of the discussion to the insulin bio-assay problem. For me the application of our observations in addition to the bio-assay of insulin like activity is really rather incidental. I am fascinated however by the physiologic implications of this so highly sensitive responsiveness of adipose tissue to insulin and I have been anxious to hear the comments of this group on this general topic.

BEIGELMAN: Evidence has been presented at this symposium that plasma insulin like activity is definitely due at least in part to insulin. However other factors may be present in plasma which have insulin like activity. Walaas *et al* in Oslo, Norway have obtained by dialysis a substance from normal plasma which appears to be amino acid or peptide is definitely not insulin and has insulin like activity. Following extraction of this material there was no residual insulin like activity in the plasma. Factors such as this may be of physiological or pathological importance.



PART VII

INSULIN ANTAGONISTS

*Chairman* PROFESSOR T RUSSELL FRASER



# INSULIN ANTAGONISTS IN DIABETIC PLASMA

J VAILLANCE-OWEN

*University of Durham Medical School  
King's College Newcastle-upon-Tyne*

The rat diaphragm technique that we have published and used for estimating plasma insulin activity<sup>6</sup> and through it any insulin antagonism in the plasma of diabetic patients is basically similar though differing somewhat in practical detail from that used by Groen<sup>4</sup> and Randle<sup>5</sup>

Probably the most important difference is in the handling of the plasma itself. We compare the glucose uptake from undiluted plasma with that achieved by the buffer solution with and without added insulin. We ordinarily find significant differences between the lowest concentration of insulin tested namely 10 micro units/ml and the basal level which is the amount of glucose taken up by the diaphragm when no insulin is added to the medium.

The index of precision ( $\lambda$ ) varies between 0.18 and 0.28. However the results are usually analysed and expressed as the glucose uptake values themselves above the basal level. This after all is the essential data from which any estimate of insulin activity can be made.

We have been particularly interested in the two broad clinical types of diabetic patient: those who require insulin treatment without which they rapidly lose weight and become ketotic; and those who do not need insulin and show no tendency to ketosis unless their diabetes is complicated by infection. The latter are usually obese and often recover from the diabetic state when they lose weight on a low carbohydrate-reducing diet.

Insulin activity was found in the plasma of all these obese patients. Also as with normal subjects when insulin was added *in vitro* to the plasma from these patients its activity was not diminished i.e. there was no measurable insulin antagonism in the plasma. This suggests that the well-known resistance to insulin which these patients exhibit resides in the tissues.

On the other hand in uncontrolled but non-ketotic diabetic



patients who require insulin no plasma insulin activity was found. Moreover when insulin was added to the plasma from these patients its activity was apparently inhibited i.e. not fully recovered. If the patients in the group were controlled so that their blood was physiological at the time of the test then plasma insulin activity was again found essentially in the normal range and now all insulin could be recovered? (Fig. 1 and Table I). Again this antagonism could be removed by diluting the plasma prior to assay. With the plasma from the uncontrolled insulin-requiring diabetic diluted 1 in 4 then insulin activity is found (Table II).

It is important to stress that these patients were not from insulin therapy for longer than at the most 24 hours. Therefore the finding on dilution does not indicate that there is no endogenous insulin in their plasma although this may be. It only reveals the activity of the exogenous insulin which has been administered to them either 1 hour before the test was made or the previous day and is masked by the inhibitor in plasma. Also this observation would explain why the use of diluted plasma in their assay procedure have ordinarily found no significant difference in plasma insulin activity in normal and diabetic subjects.

This antagonism to insulin found in the plasma of insulin-requiring diabetics may be an exaggeration of the antagonism found in normal plasma, where the antagonism is completely overcome by adequate insulin production by the pancreas. In normal plasma the antagonism can be revealed by pancreatectomy.<sup>8</sup>

Also we have recently found that the antagonism of these patients resides in the albumin fraction of the plasma and that similar antagonism is present in the plasma fraction from normal subjects. However the albumin from normal subjects is less active in this respect.<sup>9</sup>

In this study the freeze-dried fractions obtained in buffer solution containing glucose and insulin activity were assayed alone and to the buffer with dissolved fraction to determine the concentration of 1000  $\mu$ u/ml.

#### FRACTIONATION OF DIABETIC PLASMA PROTEINS

As was found previously full saturation of the plasma with sodium chloride precipitated the antagonism. The active precipitate did not contain

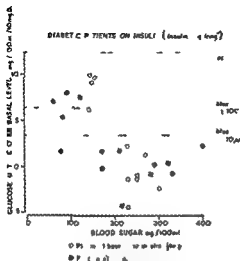


FIG. 1. Plasma insulin activity in insulin requiring diabetic patients shows no difference between those with elevated and physiological blood sugar.

TABLE I

1. SUMMARY OF EXPERIMENTS ON PLASMA INSULIN ACTIVITY AND INSULIN ANTAGONISM IN DIABETIC PATIENTS

Mean glucose uptake above the basal level  $\pm$  standard error of the mean (mg/100 ml/100 mg dry wt & plasma)

Group	Plasma	Plasma after glucose	Plasma + 400 $\mu$ /ml insulin
Obese diabetics	$6.97 \pm 0.350$	$9.53 \pm 0.99$	$10.0 \pm 0.40$
Insulin-requiring diabetics with elevated blood sugar	$-0.56 \pm 0.50$		$1.70 \pm 0.623$
Insulin-requiring diabetics with physiological blood sugar	$7.24 \pm 0.893$		$9.74 \pm 1.677$

In the insulin-requiring group with elevated blood sugar whether the plasma was taken alone or with 400  $\mu$ /ml. of insulin added the uptake values do not differ significantly from zero—indicating no measurable insulin activity. Whereas, in the same group when the blood sugar is physiological there is a highly significant increased uptake above zero with the plasma alone and then added insulin can be almost fully recovered as is also found with the obese diabetic group.

contain a substantial amount of albumin. Owing to the poor fractionation of the plasma proteins by this method sodium sulphate was employed in subsequent experiments. The activity of the fractions obtained by sodium-sulphate fractionation of diabetic plasma is shown in Table III. This shows that the precipitate obtained by saturation with 18 per cent sodium sulphate was devoid of antagonistic activity whereas the supernatant had such activity.

Paper electrophoresis showed that the sodium-sulphate fractionation effected a complete separation of the  $\gamma$  globulin and albumin fractions: i.e. all the  $\gamma$  globulin was in the precipitate and all the albumin in the supernatant. The other globulin components of the

TABLE II

EFFECT OF DILUTING PLASMA FROM INSULIN REQUIRING DIABETICS

When the plasma is undiluted there is no significant glucose uptake above zero indicating no measurable insulin activity whereas when the plasma is diluted 1:4 there is a highly significant increased uptake above zero indicating insulin activity in the plasma

Group and (No.)	Mean glucose uptake above the basal level is standard or of the mean (mg/100 ml/10 mg d. cohragm)	
	Undiluted plasma	Diluted 1/4
Insulin requiring diabetics with elevated blood sugar (4)	$-0.37 \pm 0.77$	$5.47 \pm 0.43$

plasma were present in both fractions. It was concluded that the antagonistic activity was not associated with the  $\gamma$ -globulin fraction.

Attempts to fractionate the antagonistic activity of the supernatant from 18 per cent sodium sulphate by higher concentrations of sodium sulphate were not successful (Table III). These results were consistent with the idea that the activity was associated with the albumin in the diabetic plasma. Albumin was, therefore prepared from diabetic plasma by the TCA-ethanol method of Debro *et al.*<sup>2</sup> As will be seen from Table III this albumin antagonized the effect of insulin. The inhibition of the added insulin was complete when the concentration of albumin in the buffer was between 3.5 and 5.5 per cent which includes the physiological range. When the concentration was reduced to 1.25 per cent there was still marked antagonism of the added insulin. Paper electrophoresis of these albumin fractions failed to reveal any other plasma protein.

# J VALLANCE-OWEN

## FRACTIONATION OF PROTEINS FROM NORMAL PLASMA

The demonstration of the presence of an insulin albumin from diabetic plasma prompted us to test similar protein fraction from normal plasma. As Table IV this normal albumin in physiological concentration had antagonistic activity. However unlike albumin plasma a concentration of 1.25 per cent failed to show ism. The possibility that this activity of normal plasma due to a change in the albumin associated with the

TABLE III  
INSULIN ANTAGONISM OF FRACTIONS FROM DIABETIC PLASMA  
(number of observations in parenthesis)

Fraction	$\frac{\text{Mg glu septic b has l les}}{\pm \text{ standard error fish m e n}}$ $(\text{mg/100 ml/10 mg d pl t gm})$	
	$\frac{\text{B f f} +}{1000 \text{ m/ml ml}}$	$\frac{\text{I c t n n h}}{1000 \text{ u/ml ml}}$
Precipitate from 18 per cent sodium sulphate	$12.70 \pm 0.91$ (4)	$13.17 \pm 1.54$ (4)
Supernatant from 18 per cent sodium sulphate	$12.68 \pm 0.64$ (6)	$13.02 \pm 1.17$ (6)
a) Precipitate from 22 per cent sodium sulphate b) Supernatant from 22 per cent sodium sulphate	$12.43 \pm 1.02$ (9)	$13.10 \pm 1.14$ (9)
Albumin		
1.25 per cent	$12.22 \pm 1.08$ (4)	$13.17 \pm 0.23$ (4)
1.25 per cent	$12.22 \pm 1.05$ (4)	$13.15 \pm 0.67$ (4)

method of preparation was tested by examining the activity of two samples of ether-fractionated albumin. As shown in the table there also inhibited the action of insulin. One sample of ethanol-fractionated plasma albumin (Cohn Fraction V) had similar activity. The possibility that the antagonistic activity was associated with a substance incapable of precipitation with TCA e.g. a polysaccharide was excluded by changing the method of preparation of the albumin. Thus when the plasma proteins were precipitated with 5 per cent TCA and the albumin extracted from the washed precipitate with ethanol this albumin was fully active.

Since the albumin from normal plasma inhibited the action of insulin and yet the whole normal plasma did not exhibit any such antagonism it was of interest to test the activity of fractions obtained from normal plasma by treatment with sodium sulphate. It will be seen from Table IV that in contrast with the results with diabetic plasma the precipitates obtained with 18 per cent sodium sulphate stimulated the activity of insulin and this supernatant was

TABLE IV  
INSULIN ANTAGONISM OF FRACTIONS FROM NORMAL PLASMA  
(number of observations in parentheses)

Fraction	Mean glucose uptake above basal level ± standard error of the mean (mg/100 ml/10 mg diaphragm)	
	Buffer + 1000 µu/ml insulin	Fraction in buffer + 1000 µu/ml insulin
Albumin (TCA-ethanol) 3.55 per cent	11.43 ± 0.6 (6)	0.50 ± 0.48 (6)
2.5 per cent	12.63 ± 10.4 (3)	6.60 ± 1.01 (3)
1.25 per cent	11.33 ± 0.74 (4)	11.17 ± 0.89 (4)
Albumin (ether fractionation) 5 per cent	12.50 ± 1.40 (2)	2.75 ± 0.45 (2)
Albumin (Cohn Fraction V) 3.5 per cent	12.40 (1)	2.40 (1)
Precipitate from 18 per cent sodium sulphate	11.11 ± 0.34 (4)	15.61 ± 0.70 (4)
Supernatant from 18 per cent sodium sulphate	12.42 ± 0.65 (4)	12.15 ± 0.22 (4)

devoid of antagonistic activity. Since the amount of albumin in the supernatant would be sufficient to effect complete inhibition of 1000 µu/ml of insulin, it appears that normal plasma contains a substance which the antagonistic activity of the albumin. This substance be insulin itself. It seems that the action of insulin on the  $\alpha$ -gm is the  $\beta$ -action. Firstly

the albumin in these physiological concentrations merely reduced the glucose uptake of the diaphragm to that achieved by the tissue in the absence of added insulin. Secondly the addition of large amounts of insulin i.e. 10 000  $\mu$ u/ml as above partially counteracted the antagonism of the albumin. The results of the experiments illustrating this point are shown in Table V.

Very recently we have studied three patients who have been hypophysectomized. Two had the pituitary ablated by transnasal implants of yttrium<sup>90</sup> into the sella turcica and one had the pituitary removed by surgery.

TABLE V

EFFECT OF THE ADDITION OF 10 000  $\mu$ U/ML INSULIN TO ALBUMIN FROM NORMAL SUBJECTS  
(number of observations: parentheses)

Factor	Muscle glucose uptake both basal and ± standard error of the mean ( $\mu$ g/100 ml/10 mg dry ph. gm)	
	Buffer + 1000 $\mu$ u/ml insulin	Fraction buffer + 10 000 $\mu$ u/ml insulin
Normal albumin 4 per cent*	12.33 ± 0.93 (4)	5.93 ± 0.67 (4)

\*As will be seen from Table IV albumin at this concentration completely antagonized the effect of insulin at 1000  $\mu$ u/ml.

The albumin fraction of the plasma proteins from each of these patients prepared as before was devoid of antagonistic activity<sup>10</sup> (Table VI).

Again diabetic albumin (300 mg) prepared and with the activity previously described was dissolved in 4 ml of borate-phosphate buffer and passed through a column (40 cm × 1.5 cm) of partially acetylated cellulose. The protein was eluted with buffer and a total of 150 ml of buffer was collected, the eluent extensively dialysed and the protein freeze dried. When the activity of this protein which was electrophoretically identical with albumin was tested, the original activity against insulin was found to have been removed by passage through the column.

These observations would indicate that the antagonism is probably not due to albumin *per se* but to a substance bound to it which is related in some way to the pituitary gland.

However the pituitary gland may not be solely responsible for this insulin antagonism as is suggested from studies made on experimental diabetes in animals<sup>8</sup>

Normal depancreatized depancreatized-adrenalectomized (Long Lukens) and depancreatized-hypophysectomized (Houssay) cats have been studied with and without various hormonal replacements

In normal fasting cats the mean plasma insulin activity was 114  $\mu$ u/ml and when a known amount of insulin was added to the plasma *in vitro* its activity was not diminished

TABLE VI

SUMMARY OF EXPERIMENTS ON ALBUMIN FROM HYPOPHYSECTOMIZED PATIENTS AND ON DIABETIC ALBUMIN PASSED THROUGH A COLUMN OF PARTIALLY ACETYLATED CELLULOSE

Fraction	Mean glucose uptake above basal level $\pm$ standard error of the mean (mg/100 ml/10 mg diaphragm)	
	Buffer + 1000 $\mu$ u/ml insulin	Fraction in buffer + 1000 $\mu$ u/ml insulin
Hypox albumin 4 per cent	11.85 $\pm$ 0.68 (8)	11.43 $\pm$ 0.80 (8)
Diabetic albumin through column 1.25 per cent	13.12 $\pm$ 0.65 (6)	12.58 $\pm$ 1.28 (6)

In our study of depancreatized cats the animals were handled in one of two ways. In one the blood was withdrawn 2 or 3 days after pancreatectomy. In the other the depancreatized cats were maintained on adequate amounts of soluble insulin for at least 5 days after operation. Insulin injections were discontinued for 3 days before the removal of the blood samples. In either case there was no measurable plasma insulin activity in these animals and moreover there was an inhibition of added insulin.

This inhibition could be abolished either by hypophysectomy or bilateral adrenalectomy. In either Houssay or Long-Lukens cats there was again no plasma insulin activity but now the activity of insulin added to the plasma was not diminished some 100 per cent being recovered.

Cortisone or hydrocortisone 10 mg/day injected subcutaneously

for 4 days into the Long Lukens type of animal restored the inhibiting properties of the plasma. The same dose of hydrocortisone injected into Houssay cats for 4 days and even longer until severe ketosis was produced did not restore the inhibitory activity of the plasma from these animals.

Growth hormone (3 mg/day for 4 days) injected subcutaneously into either Long-Lukens or Houssay cats failed to restore the inhibiting properties to their plasma.

TABLE VII  
SUMMARY OF EXPERIMENTS ON PLASMA INSULIN ACTIVITY AND INSULIN  
ANTAGONISM IN CATS

Group and (N)	Mean glucose uptake above the level of the plasma buffer alone $\pm$ standard error of the mean (mg/100 ml/10 mg of plasma)		
	Buffer + insulin*	Plasma alone	Plasma + insulin*
Normal (7)	12.63 $\pm$ 0.74	6.60 $\pm$ 0.73	13.33 $\pm$ 0.82
Depancreatized (3)	11.73 $\pm$ 0.50	0.37 $\pm$ 0.48	6.19 $\pm$ 0.47
Depancreatized + F (3)	11.60 $\pm$ 0.68	0.0 $\pm$ 0.09	11.10 $\pm$ 0.75
Depancreatized + F (3)	11.55 $\pm$ 0.74	0.14 $\pm$ 0.32	12.25 $\pm$ 0.63
Depancreatized + GH (4)	11.33 $\pm$ 0.35	0.8 $\pm$ 0.52	11.95 $\pm$ 1.05
Depancreatized + E (3)	12.53 $\pm$ 0.27	0.81 $\pm$ 1.03	13.22 $\pm$ 0.81
Depancreatized + E or F (3)	11.52 $\pm$ 0.64	-0.50 $\pm$ 0.82	2.57 $\pm$ 1.92
Depancreatized + GH (3)	11.80 $\pm$ 0.79	-0.50 $\pm$ 0.58	11.60 $\pm$ 0.82

\* Insulin was added to make 1000 micro-units/ml of buffer or plasma.

In the normal animal there is a highly significant increased uptake of glucose above zero but in all the other operated animals in which pancreatectomy was the common feature the glucose uptake does not differ significantly from zero indicating no remarkable insulin activity. The no significant difference between the glucose uptake in buffer plasma insulin and plasma plus insulin, except the depancreatized and the depancreatized adrenalectomized (Long Lukens) animal treated with cortisone or hydrocortisone. In these two groups there is a highly significant decrease of glucose uptake with plasma samples compared with buffer indicating the inhibition of insulin activity.

Table VII summarizes the results which suggest that both the pituitary gland and the adrenal oxysteroids must be present for insulin antagonism to be found in the plasma of depancreatized cats.

Field and Stetten have been studying the acute insulin resistance found in severe diabetic acidosis.<sup>3</sup> Using the Stadie dipping technique and measuring the glycogen deposition in the rat diaphragm they have found considerable insulin inhibitory activity in the plasma of such patients. It is evanescent often disappearing within a few hours or at least in a day or so of the commencement of insulin therapy.



This activity has been found to reside in  $\alpha_2$  of the plasma proteins and it is not a  $\beta_2$ -globulin.

This  $\alpha_2$ -globulin antagonist is probably the one that we have described.

Turning to studies made with  $^{125}\text{I}$ -insulin in New York<sup>1</sup> and Williams in Seattle<sup>2</sup>, active in this field.

It has been found that in diabetic patients phrenics who have been treated with insulin the disappearance of labelled insulin from plasma is faster than in subjects who have never been treated. The persistence of this relatively high concentration is due to binding by a factor in the plasma which satisfies the criteria of an antibody and resides in the  $\gamma$ -globulin fractions. This factor in the plasma of subjects so binds labelled insulin that it hinders its attachment to rat diaphragm and depresses its uptake by liver slices. Also bio-assay studies show that added to this binding diabetic plasma there is some of its usual hypoglycaemic effect in mice.

It is difficult to assess the importance of this in diabetes for there was no real correlation between requirements of the diabetics studied and the degree of labelled insulin in their plasma. Indeed about one-fifth treated with insulin did not exhibit any increased insulin.

Also since the majority of insulin-treated subjects are sensitive to exogenous insulin it must be supposed that the antibody complex is usually readily dissociable or that it is the pharmacological activity of native insulin.

This antagonist is clearly different from the one we have in the albumin fraction of the plasma proteins.

## CONCLUSIONS

Uncontrolled insulin-requiring diabetics have no plasma insulin activity and when insulin is added to their plasma its activity is inhibited.

If these patients are controlled however plasma insulin is found essentially in the normal range and added insulin can be recovered.

The insulin antagonism in these patients has been found to reside in the albumin fraction of the plasma proteins and is also present to a lesser extent in the albumin fractions of the plasma from normal subjects. It is not due to the albumin itself but to a substance bound to it related in some way to the pituitary gland.

This antagonist appears to be different from those described by other authors.

Finally we were told by Professor Young to let our hair down. I would like to suggest that whatever the site and mode of action of insulin in carbohydrate metabolism it has first to overcome the antagonistic activity of this substance attached to albumin. Perhaps the considerable confusion surrounding the question of insulin action on the liver may be due to this fact remembering that the liver has more than its fair share of albumin.

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# ANTIBODIES TO INSULIN

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There is a question which might be asked even at this late date and which is suggested by the subject for discussion and the question is this 'are antibodies induced by insulin?' It is true that an affirmative answer has already been given to the question still there are some reasons why it might be re-examined. For example there is good evidence to indicate that certain *in vitro* reactions which have been ascribed to insulin-anti-insulin are due instead to antigen other than insulin which may contaminate crystalline insulin and corresponding antibody<sup>5</sup>. There is also the matter of inactivation of insulin by serum from insulin resistant animals. One might ask is it possible that such neutralization is effected not by antibody but by destruction of insulin as for example by an enzyme?

I shall have something to say about these questions and I hope to show that insulin can indeed induce in animals a corresponding antibody and in addition I shall present results which have to do with different types of anti-insulins and with insulins which differ in respect of neutralization.

By antibodies to insulin I mean antibodies which neutralize the physiological action of insulin. This is the type of antibody which is encountered with certain diabetics fortunately rarely and which is responsible in these for the ineffectiveness of insulin to control the disease.

Resistance to insulin thanks to antibody formation can also be induced in animals as was first shown by Lowell and Franklin<sup>6</sup> with the rabbit.

In our work we induced antibody formation in animals of a number of species by the injection of crystalline ox or pig insulin in Freund's adjuvant.

The presence of antibodies in the sera of animals and of humans was detected by a method first used by Banting *et al*<sup>1</sup> namely inhibition by antisera of insulin induced hypoglycaemic convulsions in mice. In detail starved mice were injected with serum or

dilutions of serum mixed with an amount of insulin which when injected with saline or with normal serum caused convulsions in 90-100 per cent of the mice. Table I gives results of a typical test.

Three groups of mice were injected as shown. Each mouse received the same amount of insulin namely 0.05 unit. Each mouse

TABLE I  
MOUSE NEUTRALIZATION TESTS

Mixture injected per mouse		Number mice injected	Number convulsions	Comment
Units of insulin	Volume and dilutions of serum			
0.05	+ 0.25 ml. undil.	6	0	Insulin neutralized
0.05	+ 0.25 ml. 1/2 dil.	6	6	Insulin not neutralized
0.05	+ 0.25 ml. 1/4 dil.	6	6	Insulin not neutralized

received the same volume of serum or of diluted serum, viz. 0.25 ml. The mice which received undiluted serum showed no convulsions all other mice showed typical convulsions. That is 1 ml. of undiluted serum was capable of neutralizing 0.2 unit of insulin but was not capable of neutralizing 0.4 unit.

TABLE II  
NEUTRALIZATION OF INSULIN WITH ANTISERA

Anti-insulin (origin)		Insulin (extracted from pancreas of)
Guinea pig	} Neutralizes	Pig (Swine)
Rabbit		Ox
Sheep		Rabbit
Horse		Sheep
Human		Horse
		Monkey (macaca rhesus)
		Chicken
		Fish (Larg)
		Whale
		Human

Table II gives a summary of some neutralization results.

Anti-insulin had been induced in the guinea-pig, the rabbit, the sheep and the horse by a series of injections of crystalline ox or pig insulin in Freund's adjuvant. The human serum was from a diabetic who had developed resistance to insulin.

The neutralizing capacity of each serum was tested against each of the extracted insulins shown in the second column by the convulsion-inhibition test. It was found that each antiserum neutralized each of the insulins shown. That is antiserum from the guinea-pig neutralized not only pig or ox insulin which had been used to induce anti-insulin but also insulin from the rabbit sheep horse monkey chicken fish (ling) whale and human and so with antiserum from the rabbit and the sheep and the horse and the human. And note that antiserum from the rabbit neutralized insulin extracted from rabbit pancreas and similarly antiserum from the sheep the horse and the human neutralized insulin extracted from the pancreas of the sheep the horse and the human respectively.

There were good reasons for the assumption that neutralization of insulin by antiserum was an antigen-antibody phenomenon. For example just as with antibodies in general anti-insulin had been induced by a series of injections of a protein in this case insulin neutralizing activity was associated with the globulin fraction of the antiserum and finally insulin could be recovered from a neutral insulin-anti-insulin mixture by extraction with acid alcohol thus indicating that inactivation was not due to destruction.

Since it appeared probable that one had to do here with an antigen-antibody system the possibility of *in vitro* reaction was explored and in particular the possibility of precipitation or flocculation in mixtures of insulin and anti insulin. In all preliminary observations which were made using anti insulin derived from the guinea-pig rabbit sheep and horse precipitation was not observed. Finally however in mixtures of insulin and certain specimens of serum from a resistant horse typical flocculation occurred.

A summary of the course of immunization of the horse which yielded flocculating serum is given in Table III.

Lots A, B, C and D consisted of relatively large amounts of serum 1 litre or more. Flocculation occurred with mixtures of insulin and serum of lots C and D but not with serum of lots A and B.

I shall give very briefly results on flocculation from a paper which has been submitted for publication.<sup>8</sup>

Results in Table IV have to do with the determination of the equivalence zone of insulin-anti insulin.

Each flocculation mixture consisted of a constant amount of flocculating serum (0.2 ml.) plus the amount of insulin shown in the first column in a total volume of 0.7 ml. pH 7.4. Equivalence zone

was determined by standard procedure namely two sets of mixtures as in the first two columns were prepared After flocculation had taken place all mixtures were centrifuged and supernatants poured off To one set of supernatants a constant amount of anti insulin as shown was added and to the other set a constant amount

TABLE III  
RESPONSE OF HORSE NO 2589 TO INJECTIONS OF INSULIN

Time (days)	Units of insulin injected	Potency of serum (units insulin neutralized per ml serum)
0	6.50	
28	6.50	
33		0.4
47		0.2
49	62.50	
61		0.8 Lot A
70	62.50	0.8 Lot B
85		0.8
92	62.50	0.8
105		0.8 Lot C
112	6.50	0.8
134		0.8 Lot D

of insulin Flocculation results with these are shown in the last two columns The equivalence zone is that in which there is excess of neither insulin nor anti-insulin

From washed floccules of the equivalence zone we were able to recover insulin and anti-insulin in good yields The recovered insulin and anti-insulin were assayed by mouse convulsion technique

TABLE IV  
EQUIVALENCE ZONE OF INSULIN ANTI INSULIN

Units of insulin in 0.5 ml	Anti serum C ml	Flocculation	Flocculation in supernatants	
			Plus 0.025 ml of serum C	Plus 0.25 units insulin
0.46	0.2	+	—	+
0.58	0.2	+	—	+
0.71	0.2	+	—	+
0.89	0.2	+	—	tr
2.0	0.2	+ 1st	—	—
2.2	0.2	+ 2nd	+	—
2.4	0.2	+	+	—
2.7	0.2	+	+	—
3.1	0.2	+	+	—

and not by flocculation. Here then is evidence that typical antigen-antibody flocculation may occur with insulin and anti-insulin.

This conclusion is supported by other evidence.

Table V shows graphically the result of an agar diffusion test.

TABLE VI

EFFECT OF ANTI-INSULIN ON ENDOGENOUS INSULIN

<i>Anti-insulin (or g.)</i>		<i>Endogenous insulin</i>
Guinea pig	Neutralizes	Mouse
Horse	Does not neutralize	Mouse
Human	Does not neutralize	Mouse
Mouse	} Endogenous insulin of anti-insulin producer not neutralized	
Guinea pig		
Rabbit		
Sheep		
Horse		
Human		

The insulin and anti-insulin solutions were equal in volume and were in equivalence amounts. The position and curvature of the line of precipitation is consistent with a smaller molecular species insulin diffusing towards a larger molecular species anti-insulin (horse globulin).



There is a further piece of evidence which indirectly supports the conclusion that flocculation is due to insulin and anti-insulin and which depends on the following considerations. Boyd and Hooker<sup>9</sup> derived a formula for the relation between the ratio of antibody to antigen in floccules at the equivalence point and the molecular weight of antigen.

The ratio of anti-insulin to insulin in the equivalence zone was calculated from total nitrogen of floccules and an estimate of the amount of insulin precipitated.

When this ratio was used in the formula of Boyd and Hooker a value for the molecular weight of insulin of 24,000 to 36,000 was obtained. This value is in the order of magnitude of the molecular weight of insulin in aqueous solution pH 7 to 8.

There are different types of neutralizing antibodies. These for example can be flocculating or non-flocculating. Indeed the flocculating serum with which we worked contained in addition to flocculating antibodies non-flocculating antibodies as we were able to show by fractionation with ammonium sulphate. Other differences in antisera are shown in Table VI. Here are three neutralizing antisera viz guinea-pig, horse and human, all of which were produced by injection of crystalline ox or pig insulin. Only the anti-serum from the guinea-pig neutralized mouse-endogenous insulin as indicated by the production of signs of diabetes in the mouse. In this connection some work of Lacy and Davies<sup>4</sup> is of interest. These investigators labelled with fluorescein isocyanate a globulin fraction of guinea-pig serum containing anti-insulin. The labelled anti-insulin globulin was used to stain frozen sections of pancreas from beef, mouse, guinea-pig, rabbit and man. A yellow green fluorescence was produced in the islets of mouse and ox pancreas which was inhibited by prior addition to the antiserum of crystalline pork insulin. Fluorescence was not produced in the islets of guinea-pig, rabbit or human pancreas. Results with mouse and guinea-pig pancreas and these are the only two for which we have relevant data are consistent with our findings, namely that antiserum of the guinea-pig neutralized endogenous mouse insulin but did not neutralize endogenous guinea-pig insulin.<sup>4</sup>

Also in Table VI there are indicated differences in insulins in respect of neutralization. The endogenous insulin of the anti-insulin producer is not neutralized. Whereas for example anti-insulin from the horse neutralized insulin extracted from horse

pancreas it did not neutralize the endogenous insulin of the horse-producing anti insulin since the animal was healthy and showed no signs of diabetes and so for the antisera of the rabbit and the sheep and even the human. This latter was a severe diabetic woman whose diabetes could not be controlled by insulin nor by an oral preparation. The disease however was controlled by a low caloric diet and on this regimen the patient successfully withstood the amputation of a leg which had to be done because of a gangrenous foot.<sup>3</sup>

Table VII is also concerned with differences in insulins. This table gives a summary of properties of guinea-pig insulin.

TABLE VII  
GUINEA-PIG INSULIN

- 1 Mixture of guinea pig and ox insulins may be separated by partition chromatography
- 2 Guinea-pig insulin not neutralized by anti-insulin (mouse conjugation test)
- 3 Guinea pig insulin lowers blood sugar of mouse injected with anti-insulin from guinea pig
- 4 Guinea-pig insulin does not lower blood sugar of anti-insulin producing guinea pig

Since guinea-pig insulin is not effective in lowering the blood sugar of a guinea pig in which antibodies have been induced by ox or pig insulin it is altogether improbable that it would be useful for the treatment of a diabetic who has developed antibody-type resistance to injected insulin.

Table VIII shows a comparison of neutralization of ox and cod insulins.

TABLE VIII  
COMPARISON OF COD AND OX INSULINS

Anti-insulin (horse)	Factor used	Anti-insulin (horse)	Factor used
	Ox insulin (0.05 u per mouse)		Cod insulin (0.03 u per mouse)
Undil.	0/12	Undil.	2/12
1/2	0/12	1/2	7/12
1/4	1/12	1/4	8/12
1/8	12/12	1/8	7/12
Saline	12/12	Saline	8/12

These are different Evidence from flocculation and neutralization<sup>8</sup> indicates that soluble ox insulin-anti-insulin complex is dissociated Results here indicate that soluble cod insulin-anti insulin complex has a larger dissociation constant than ox insulin anti-insulin

It would be interesting to know why endogenous insulins are not neutralized by antibodies which neutralize insulin extracted from pancreas of the same species And it would certainly be desirable to have available an insulin effective in insulin-resistant diabetics that is to say an insulin which is not neutralized by antibodies induced in the human by ox or pig insulin

### SUMMARY

By the injection of ox or pig insulin into animals it is possible to induce the formation of antisera which are capable of neutralizing the physiological effect of insulins extracted from the pancreas of a number of species of animals Such sera in mixture with insulin may or may not exhibit the phenomenon of flocculation A study of flocculation and neutralization with an anti-insulin serum from a horse showed clearly that neutralization of insulin by the serum was an antigen-antibody reaction It is highly probable that neutralization of insulin by non-flocculating antisera is also an antigen-antibody reaction

Antibodies to insulin may differ not only in respect of flocculation and non-flocculation but also in respect of insulins neutralized For example antiserum induced in the guinea-pig by the injection of ox or pig insulin was capable of neutralizing endogenous mouse insulin whereas anti-insulins induced in the horse human and mouse were not

Extracted insulins from the pancreas of the ox pig rabbit sheep horse monkey chicken fish (ling) and man were neutralized by antisera induced by ox or pig insulin in the guinea-pig rabbit sheep horse and man Extracted insulin from guinea-pig pancreas was not neutralized as shown by inhibition of convulsions and by blood-sugar test in mice it was however neutralized in the guinea pig carrying antibodies actively induced by ox or pig insulin Insulin extracted from cod pancreas is incompletely neutralized an effect which depends probably on mass action

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## DISCUSSION

YOUNG Dr Moloney has introduced into the subject which we are considering during these two days the ideas and methods of investigation of the immunologist and the serologist in a most important and I think entertaining fashion

The particular interest pertaining to guinea pig insulin is one that I certainly share. More than 20 years ago H P Marks and I (*Nature* 1940 146 31) prepared a small quantity of insulin from guinea-pig pancreas. We were very interested in the relatively low yields of insulin which we obtained from the pancreas of this animal. The yield per 100 g of body weight was less than one tenth of that for the other species which we were investigating at the time. This was unexpected in view of the fact that in the guinea pig pancreas there are clear granules in the  $\beta$  cells of the pancreatic islets which diminish in number when the blood sugar rises and which show many signs of being the precursors of insulin. It is possible that there is an antagonistic factor to guinea pig insulin in the pancreas itself though Griffiths who was in the laboratory with us got some evidence for this later we were not able clearly to demonstrate this. Perhaps Dr Moloney would care to comment on these observations.

With regard to the species differences of insulin the work of Sanger - which has covered a considerable number of different insulins has shown chemical differences in the amino acids within the intra-chain disulphide link of the A chain of insulin although as far as I know nothing is known about the structure of guinea pig insulin.

I was particularly glad that Dr Vallance-Owen still thinks that the pituitary gland is a going concern. It is a concern in which I myself have been interested for many years and I am still unrepentant in my belief that growth hormone is of particular importance in the antagonism to insulin which is exerted by pituitary extracts and by the secretions of the pituitary gland itself.

I noted that Dr Vallance Owen in his experiments with Dr Lukens found no clear effect of growth hormone in the Houssay animal when the growth hormone was given alone. I am not clear whether growth hormone and a small amount of adrenal steroid were also given in similar experiments. Dr Vallance-Owen, was that so?

VALLANCE OWEN No Sir Growth hormone was given alone to the Houssay animals adrenal steroids were not given in addition

YOUNG Do you think the growth hormone is concerned in the pituitary effect that you and Dr Lukens observed?

VALLANCE-OWEN Yes I do

YOUNG I am glad about that One might ask now what is the physiological significance of this antagonism between pituitary hormones on the one hand of which I think growth hormone is one and insulin on the other? This question is an important one because there is some evidence which I cannot discuss now that in some species of animals an increased secretion of insulin by the islets of Langerhans of the pancreas is stimulated by growth hormone Why one may ask should there be a stimulation of insulin secretion by the pancreas in some species at the same time as there is produced in these animals by means which are not certain and by a mechanism which is still far from clear an antagonism to the action of insulin as far as carbohydrate utilization in muscles is concerned?

I do not think any final answer can be given to this question but I still hold to the view that I have held now for quite a few years of the importance of insulin in protein anabolism The antagonistic action of the growth hormone or the insulin antagonist it elicits the effect of insulin on carbohydrate utilization may permit the effectiveness of insulin on protein anabolism to be emphasized without a danger of an undesirable fall of blood-sugar level

The variety of known antagonists to insulin is becoming rather disturbing and we cannot assume at the present time that there is any one main antagonist to the action of insulin on carbohydrate utilization There may exist a series of antagonists existing in different species under different conditions but much research needs to be done on this point at the present time

The question of the presence of insulin in blood plasma masked by antagonists is one that Dr Randle and I met some years ago when we found that in a cat which had been rendered persistently diabetic by pituitary treatment the metahypophyseal diabetes was associated with a condition in which no insulin could be detected in the plasma by the rat diaphragm method as used in our laboratory If the plasma of this diabetic animal was frozen and thawed three times and the insulin activity then measured by the diaphragm method insulin activity was found to have appeared and to be about the normal amount Is it possible therefore that in this animal with diabetes originally of pituitary origin but associated more directly and primarily with islet lesions there is insulin circulating in a form which is completely neutralized as far as action on the normal diaphragm method is concerned but which can be

unmasked by a process which might be expected to inactivate a lipoprotein? I do not know what the final answer to that question is but it again emphasizes the dangers of measuring insulin in plasma by only one method and of making any assumption that one is in fact measuring insulin itself

Some experiments carried out by Dr Whitney and myself (*Biochem J* 1957 **64** 648) some years ago are possibly relevant to this point. We investigated the effect of the intravenous injection of glucose (100 mg/ml) on the plasma insulin activity of the normal rat, and we also measured the uptake of glucose by the diaphragm of these rats given glucose intravenously. Control experiments were carried out with diaphragm from rats which were not treated with glucose.

We found as we rather expected, that when the blood sugar level had been raised by the injection of glucose the capacity of the diaphragm of the injected animal to take up glucose when the diaphragm was excised and immersed in glucose-containing buffer also rose returning to a little below the normal at about 120 minutes.

We took the plasma from these glucose-injected rats and assayed it by the diaphragm method for insulin like activity. We rather expected that the plasma insulin activity would be raised soon after the injection of glucose but in fact we found that it was very definitely lowered rising to about the normal level at 60 minutes after which it rose above normal where it remained until the end of the experimental period of 120 minutes.

I cannot believe that insulin secretion by the pancreas is not increased when the blood-sugar level rises. There is too much evidence of all sorts to allow a contrary view to be accepted. It is possible that insulin which was present in the plasma to begin with became bound in the tissues when the blood sugar level rose and there may therefore have been a removal of insulin into the tissues at such a rate that insulin replacement by secretion from the pancreas was initially unable to maintain a steady level of blood insulin. There is also the possibility that insulin antagonists were secreted into the blood stream soon after glucose had been injected but I do not know what is the simple explanation of these findings. This type of experiment emphasizes the complexity of the situation which one is dealing with in discussing the activity of insulin in plasma and it also shows that the secretion or development of antagonists to insulin might occur under physiological conditions for reasons which I think we cannot easily understand at the present time.

I personally have very much enjoyed the papers today. Particularly that in which Dr Moloney made so clear the immunological properties of the system he has investigated. The fact that his antibodies to insulin provide a valuable tool for the elucidation of the problems that we have been discussing has already been clearly illustrated by the results which

Dr Taylor at Cambridge and Dr Wright in London put before us earlier this morning. Undoubtedly Dr Moloney has placed an extremely valuable and powerful tool in the hands of those who are interested in these problems.

PETROW It has been shown in a limited number of publications that growth hormone appears to stimulate resistance to infection of animals through some adrenal mechanism. I just wondered whether to the extent to which antibodies are involved in that effect you might not have a partial explanation of growth hormone antagonism to insulin through an antibody mechanism of this sort.

YOUNG Dr Petrow I presume you are referring to Selye's work here. I do not know that there is any clear evidence of the interpretation which you suggest. It is a possible one but I prefer to reserve judgment on this aspect of the problem.

The effects of growth hormone on the adrenal cortex of the hypophysectomized rat were investigated by Stack Dunne *et al* (*J Endocrinol* 1955 12, 174) in my laboratory some years ago. There is no clear indication that growth hormone stimulates the secretion of adrenal steroids. There is some stimulation of mitosis in the adrenal cortex but I am very hesitant about the interpretation of much of this work at the present time.

FISHER I do not think that in view of the complexity of the situation that has been disclosed here that the injection of another possible complexity can do much harm. However one thing which occurs to me about a great deal of this assay work and the effect of protein fractions is that the assumption seems to be inherent that the diaphragm itself is invariant. This sort of effect and some of the stimulant effects which Dr Vallance-Owen has described could be the result of non-specific or other specific effects of serum proteins on the diaphragm metabolism.

I am reminded of some work which Krease has just published showing that the diaphragm, however carefully prepared, tends to lose potassium fairly rapidly and that the only way he can prevent that is by the addition of various serum protein fractions. It has been known for a long time — D. K. Hill was the first person to do it — that an increase in potassium concentration extracellularly in muscle has a very profound effect on this metabolism.

These are considerations which might be borne in mind before one is quite sure that these effects are specific insulin-stimulating or insulin-inhibiting.

STEWART May I just say something about the point which Professor Young has brought up on the insulin content of the guinea pig pancreas? We have devised a technique for measuring the insulin content of acid alcohol extracts using the mouse convulsion method. If we feed mice on a carbohydrate diet and pre-treat them in the fasted state with Phenergan

they are extremely sensitive to insulin. We find that using this technique the guinea pig has between 1-1½ units per gramme fresh weight of pancreas compared with 2-5 units per gramme for the rabbit so the difference is not all that great, and our values are much higher than those found in the literature. We think it is something to do with the actual working up of the insulin from the pancreas of the guinea pig which may be quite different from that of working up insulin from ox pancreas.

OAKLEY With regard to the detection of insulin antibodies Dr Cunniffe has been using a test at King's which is as yet unpublished in this connection but in our hands it does seem to give so far encouraging results. What he has been doing is a passive cutaneous anaphylaxis test which consists of giving intradermal injections into a guinea pig of the serum which one is investigating - these of course can be done in varying dilutions - and 24 hours later injecting into one hind leg 1 ml of pontamine blue and 80 units of crystalline soluble insulin into the other hind leg. If insulin antibodies were present you would get a rapid formation of a circumscribed blue ring or area around the site of injection of the serum containing the antibody.

It is a relatively simple test in our hands which has so far given remarkably consistent results although we have not been using it long enough. However in thirty or forty controls they have all been negative and in thirty or forty diabetics not on insulin there has not been a single positive. In large series of diabetics on insulin we have had two weak positives in patients whose insulin requirements have been just about 100 units of soluble insulin a day and with eleven patients whose insulin requirements have been between 500 and 1,000 units of insulin a day we have had a strong positive result in every case.

SCHAMBYE I just want to ask Dr Vallance-Owen and Dr Taylor if they would like to comment on the fact that in Dr Taylor's experiments where he used labelled insulin he found all the insulin in the last part of the albumin fraction in the  $\alpha$  and  $\alpha_2$ -globulin fraction. Is it possible that the albumin factor of Dr Vallance-Owen plays any role in the determination of the insulin activity in the fraction which Dr Taylor studied?

WRIGHT I would like to ask Dr Moloney whether he has tested any insulin antiserum produced in rabbits by Drs Arquillo and Stavitsky. Have tested the action of a specimen of such serum in high concentration upon the effects of insulin on the isolated rat diaphragm and have found that even in high concentration it has no effect.

MOLONEY Anti insulin produced in the rabbit by Stavitsky, Arquillo and in the guinea pig and sheep by us were assayed in Cleveland by the Stavitsky and Arquillo *in vitro* method and in Toronto by the convulsion inhibition method. Results in Cleveland were rabbit ser



effective at  $1/64$  dilution guinea pig serum at serum at  $1/4$  results in Toronto were ■ ~  
 vented convulsions at  $1/2$  dilution rabbit serum action that is to say the results of the assays were

TAYLOR In answer to Dr Schambye may I say our albumin by fractional precipitation, insulin with it As I understand it Dr Vallance-Owen al be present in some of his albumin fractions of course with his insulin antagonist Effects on  $\epsilon$  then very likely represent our balance between ins<sup>r</sup> both present together in the albumin fraction

PART VIII

MECHANISM OF ACTION OF INSULIN AND  
OTHER HYPOGLYCAEMIC SUBSTANCES

*Chairman* PROFESSOR C. DE DUVE



# THE PERIPHERAL UPTAKE OF C 1: RELATIONSHIP TO THE MODE OF AC: AND OTHER HYPOGLYCAEMIC ST

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This work was undertaken to discover whether or not tolbutamide possess a glucose utilization any way from non-diabetics on the one hand and moderate to low doses of insulin on the other. The not respond to tolbutamide. The absence of ket observed in those diabetics responsive to sulphonyl that their own pancreas secreted sufficient insulin to . We considered that a positive increase of a capillary after the oral administration of glucose would , presence of endogenous insulin.

The procedure adopted was as follows: all subjects a .00 carbohydrate diet for 7 days prior to the test been stabilized on tolbutamide this was replaced by p' the week prior to the test. The injection of insulin in normally required it was stopped 36 hours prior to the subjects fasted overnight. On arrival they were kept for in a quiet temperature-controlled room. Capillary blood specimens were obtained in heparinized tubes as Fig 1.

A single stab into the tip of the index finger with a Ba scalpel ensured free flow of blood throughout the. Venous blood samples were obtained without the use of a syringe from the same vein in the median cubital fossa.

The blood-sugar concentrations were determined in quadruplicate by the anthrone method. The standard error of a single determination was  $\pm 1.56$  per cent. Capillary blood samples were 5 minutes prior to and after the obtaining of the venous. The mean of these two capillary blood sugar concentrations used as an estimate of the capillary blood sugar at the time the

sample was obtained. Twenty minutes after the commencement of the test 30 g of glucose per square metre body surface were then administered in 200 c.c. of lemon-flavoured tap water and the test then continued for another hour.

Persons attending a diabetic clinic were divided into four groups. Group one contained the good responders to tolbutamide. Those in group two were termed moderate responders to tolbutamide — they were those patients who showed initial response but later on a partial relapse. Group three contained the non-responders to tolbutamide who however never showed ketosis but who required

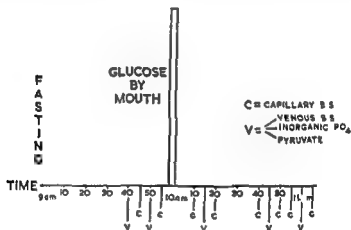


FIG. 1 Glucose utilization test

small to moderate amounts of insulin. Diabetics with a history of ketosis who required insulin constituted the fourth group. A fifth group of non-diabetic subjects was included in the trial to serve as controls.

The mean capillary venous difference prior to glucose administration was subtracted from the mean value of the differences estimated during 1 hour after glucose administration. This final value was taken to be the measure of one of the effects of endogenous insulin. Fig. 2 shows the results of these tests in the five groups of subjects. Of the diabetics the group responding to tolbutamide showed the best uptake after glucose but even so this was only approximately one-half that of the group of non-diabetic controls.

There was no significant difference between moderate responders and non-ketotic non-responders to tolbutamide but the difference between non ketotic non responders and good responders was significant. The ketotic diabetics showed a negative uptake. Among those non-diabetics initially chosen for the test were two with disseminated sclerosis two others with presumptive cortical or pituitary dysfunction. These are shown in Fig 3 and are characterized by showing a negative uptake

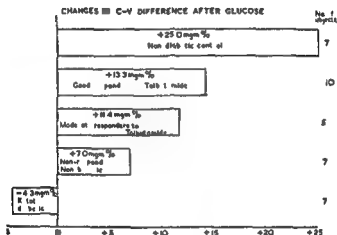


FIG 2

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Persons attending a diabetic clinic were divided into four groups. Group one contained the good responders to tolbutamide. Those in group two were termed *moderate responders* to tolbutamide—they were those patients who showed initial response but later on a partial relapse. Group three contained the non-responders to tolbutamide who however never showed ketosis but who required

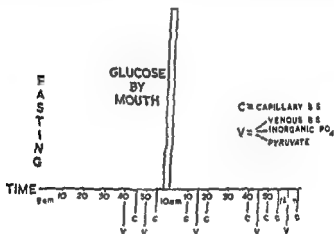


FIG. 1. Glucose utilization test.

small to moderate amounts of insulin. Diabetics with a history of ketosis who required insulin constituted the fourth group. A fifth group of non-diabetic subjects was included in the trial to serve as controls.

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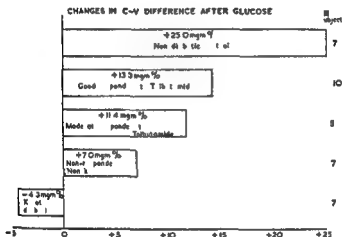


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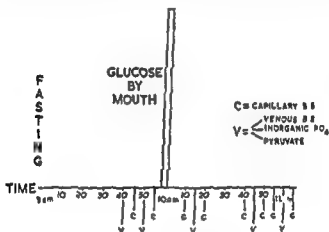


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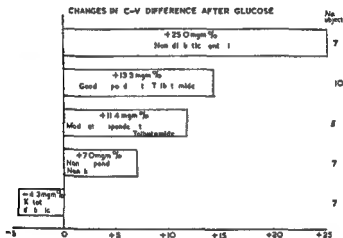


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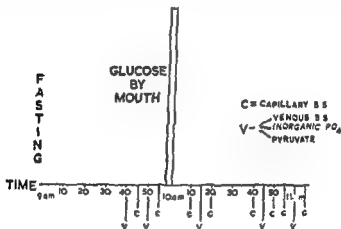


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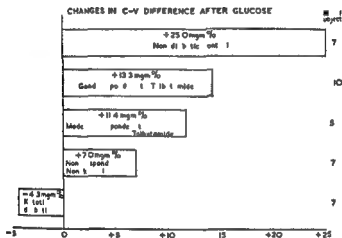


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Our results indicate that good responders to tolbutamide have a greater uptake of glucose than insulin-requiring diabetics and at the same time only approximately half the uptake of normal subjects. This suggests to us that tolbutamide responders have more endogenous insulin than those who do not respond. This has been confirmed by the plasma insulin assays of Vallance-Owen and others. The tolbutamide-sensitive diabetics do not appear however to have so much insulin available as other non-diabetic subjects. This may be due to less insulin being available in the pancreas to a

NEGATIVE C-V CHANGES IN SELECTED NON-DIABETIC SUBJECT

Case	Diagnosis	Initial capillary blood sugar mgm %	Initial C-V difference mgm %	Mean C-V difference glucose mgm %	Change in C-V difference mgm %
1	Di semi ins d Sci role	127	58	17	-41
2	Di semi ins d Sci role	72	8	-5	-13
3	Di mi o t i b Sy d m	65	-4	-23	-17
4	Gross obesity f l i g m m g i t i	93	3	1	-2

FIG. 3

slower release from the pancreas or to a greater rate of inactivation in the liver. The finding of a negative glucose uptake both in ketotic diabetics and in certain normal subjects suffering from neurological disorders or ketotic dysfunction remains to be explained.

It is of particular interest that six out of seven patients who had a history of ketosis had a negative glucose uptake. We have no evidence whether this was due to the presence of some abnormal substance in the blood such as ketones or was due to simple passive pooling or stagnation of glucose on the venous side of the circula-

Observations on the occurrence of negative differences have been reported for many years. At first thought to be due to technical errors but improved chemical techniques have confirmed them but have not yet clarified our knowledge of their causation.

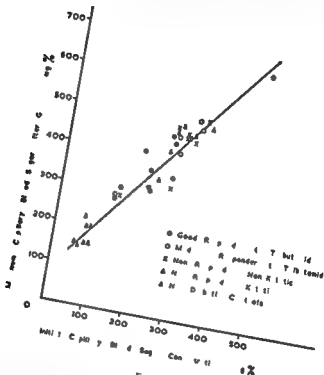


FIG 4

If the major function of the sulphonylureas is thought to be to stimulate or release endogenous insulin it would have been thought that its administration would alter the standard insulin sensitivity test only in those patients who still have a reserve of their own insulin. Fig 5 demonstrates the absence of the effect of carbutamide on insulin sensitivity tests of a young brittle diabetic with an 11 year history of the illness. Fig 6 reviews the effect of carbutamide.

## MECHANISM OF ACTION OF INSULIN

Mrs H age 56 Weight 45 kg. Feb 1957 Plot.

A IV Insulin 0.3u/kg.  
B = IV Insulin + BZ55  
C = BZ55 only

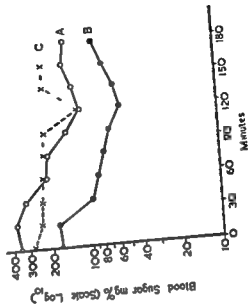


FIG. 6

Miss L age 18 Weight 60 kg. Jan Feb 1957 Plot.

A ○ IV Insulin 0.3u/kg  
B ● " " " "  
C — Insulin and DB60 (1st week)  
D - - - " " (2nd week)  
E ▲ " " (3rd week)

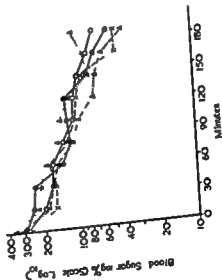


FIG. 5

on the insulin-sensitivity test of a middle-aged diabetic Jamaican lady with an 8-year history of the illness. She omitted insulin at her own volition for 6 months prior to the test. A temporary lowering of the glucose plateau is shown in the sensitivity to insulin. This patient eventually achieved stabilization with insulin for permanent control. Fig. 7 shows an apparent increase in sensitivity to insulin during tolbutamide administration in a thin young woman of 24 who had had diabetes diagnosed only a few days prior to the test procedures and

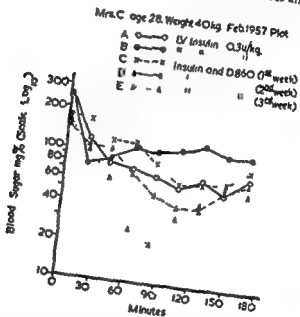


FIG. 7

never before received insulin. The apparent increase in sensitivity to injected insulin over 3 weeks while on tolbutamide did not imply however that she might be controlled solely with the latter substance. Such an attempt in fact failed and she required permanent insulin treatment for adequate stabilization. It is mere speculation to wonder whether the temporary increase in sensitivity was due to remnants of endogenous insulin being



stimulated by tolbutamide remnants however insufficient to ensure clinical control as shown by her eventual need of insulin

We are not able to separate this type of response from that of a true sparing of exogenous insulin as long as plasma insulin assays cannot differentiate between exogenous and endogenous insulin

## SOME PERIPHERAL EFFECTS OF INSULIN AND OTHER HORMONES IN MAN

JEAN GINSBURG H-J B GALBRAITH and A PATON

*Department of Medicine St Thomas's Hospital Medical School London*

The fact that insulin has a marked action on extrahepatic tissues has been shown by many workers. An increased uptake of glucose under the influence of insulin has been demonstrated in isolated tissues (Burn and Dale<sup>8</sup> Gemmill<sup>17</sup> Hechter *et al*<sup>19</sup> Hepburn and Latchford and Stadie *et al*<sup>20</sup>) and eviscerated preparations (Best *et al*<sup>5</sup> Burn and Dale<sup>8</sup> and Mann and Magath<sup>23</sup>). The widening of the arterio-venous glucose difference which occurs after intravenous or intra arterial injections of insulin in animals and man (Andres and Zierler<sup>1</sup> Bill and Burns<sup>4</sup> Cori and Cori<sup>9</sup> and Frank *et al*<sup>11, 13</sup>) has also been taken as evidence of a peripheral effect.

In the present investigation the effects of insulin hydrocortisone and glucagon have been measured in the forearm of healthy adults. Peripheral responses to insulin were also studied in patients with diabetes acromegaly and other diseases associated with disturbances of carbohydrate metabolism and with an altered sensitivity to insulin.

The forty-six subjects of the initial study were mainly medical students and colleagues; nine convalescent patients with no evidence of metabolic or endocrine disturbance were also included in this group. All subjects were tested at rest in the laboratory after a fast of 6 to 16 hours. Simultaneous samples of arterial and venous blood were taken through needles maintained throughout the test in the brachial artery and antecubital vein of one arm and in the corresponding antecubital vein of the opposite arm. Blood glucose was estimated by a modified Shaffer Hartmann method (Haslewood and Strookman<sup>18</sup>). In some tests hand or forearm blood flow were measured at half minute intervals using venous occlusion plethysmography (Barcroft and Swan<sup>2</sup>). It was thus possible to evaluate the influence of these hormones on glucose uptake in the

## PERIPHERAL EFFECTS OF INTRA-ARTERIAL INSULIN (Figs 1 and 2)

After duplicate control samples had been taken 2 units of insulin diluted with isotonic saline to a volume of 2 ml were rapidly injected into the brachial artery. Further samples were taken 5, 15, 30, 45 and 60 minutes after the injection in all subjects and at 90 and 120 minutes in a few.

The concentration of glucose in venous blood draining from the injected limb fell progressively after the injection of insulin to a mean value of only 35 mg per 100 ml at 30 minutes. One hour after the insulin the mean level in this vein had risen slightly but was still 20-30 mg below control values. When samples were taken 1½ to 2 hours after the intra-arterial injection of insulin glucose concentration in the *ipsi-lateral draining vein* was still well below control levels although glucose concentration had regained control values in arterial blood and blood from the *contra-lateral vein*.

There was an initial fall in the glucose concentration of arterial blood and venous blood from the *contra-lateral arm*. These changes were however much less than in the *ipsi-lateral draining vein* and control values were regained by the end of an hour at a time when the glucose content of venous blood from the injected limb was still below 60 mg per 100 ml.

The selective and sustained fall in glucose concentration in the *ipsi-lateral vein* is due to the direct action of insulin and provides evidence that the hormone can be irreversibly fixed in healthy tissues *in vivo* — in isolated preparations (Stadie *et al*<sup>26</sup>). Fixation of insulin must have occurred with extreme rapidity for the injection was made in less than 2 seconds and the hormone could only have been in contact with the tissues of the forearm for a very few seconds.

The effect of intra-arterial insulin was not confined to the tissues into which it was injected for the concentration of glucose also fell in arterial blood and in venous blood from the opposite arm though to a lesser extent. These changes in glucose concentration presumably reflect the peripheral action of insulin after recirculation but may also result from the action of insulin in areas other than the extremities.

There was no general or selective change in hand or forearm blood flow after intra-arterial injections of insulin. Changes in arterio-venous glucose difference in the injected arm can therefore be used as a measure of the effect of intra-arterial insulin on glucose

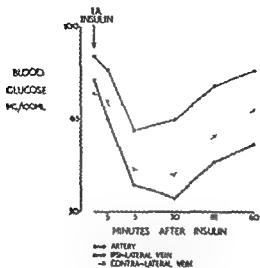


FIG. 1. Effect of an intra-arterial injection of insulin (1 unit) on arterial and venous glucose concentration as mean of fourteen experiments.

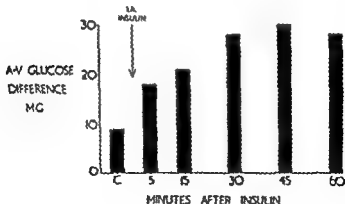


FIG. 2. Arterio-venous (A-V) glucose differences in the injected arm after an intra-arterial injection of insulin (1 unit) mean of twenty-two experiments.

uptake in that limb. Initially (Fig. 2) the arterio-venous glucose difference averaged 7 mg in both arms. Five minutes after intra-arterial insulin the arterio-venous difference in the injected arm had increased to nearly 20 mg and remained elevated for a further hour at least.

A selective increase in glucose uptake on the injected side could similarly be demonstrated when changes in the arterio-venous difference were expressed as a function of the arterial level of glucose using the formula  $A-V/A$  (Elick *et al*<sup>10</sup>).

#### PERIPHERAL EFFECTS OF HYDROCORTISONE (Figs 3 and 4)

The peripheral action of hydrocortisone was investigated in a similar manner. Hydrocortisone was given *intra-arterially* in doses of 1-25 mg. Samples were taken from the brachial artery and ante-cubital veins and blood flow was measured as before. There was no change in glucose concentrations or blood flow for 1 hour after the injection. It was therefore not possible to demonstrate any direct or immediate influence of hydrocortisone on glucose uptake in the forearm.

Similarly (Fig. 3) when 60-90 mg hydrocortisone were infused intravenously over 75 to 90 minutes there was no appreciable change in arterial glucose; a slight increase in venous glucose was however recorded by the end of the hour when over 50 mg had been infused.

The possibility that hydrocortisone might influence the action of insulin apart from any primary effect on the level of circulating glucose was also investigated.

Hydrocortisone (0.6-1 mg/min) was given intravenously for 90 minutes. Control samples were taken during the first 20 minutes of this infusion. Two units of insulin were then injected into the brachial artery and samples taken as before for a further hour.

The general pattern of response to *intra-arterial* insulin was much the same when hydrocortisone was infused (Fig. 4) as when insulin was given alone (Fig. 1). The mean changes in arterial glucose and arterio-venous glucose differences in the injected arm were not significantly different in the two groups. Thus systemic administration of over 50 mg hydrocortisone did not affect the peripheral action of insulin or modify its fixation in forearm tissue.

The prolonged administration of hydrocortisone or adrenocortical extracts undoubtedly cause a rise in blood sugar and altered

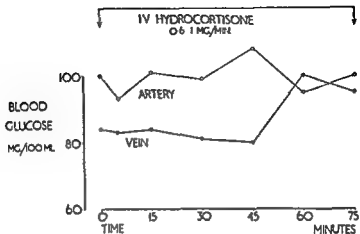


Fig. 3 Blood glucose concentration during an intravenous infusion of hydrocortisone (0.6-1 mg/min) mean of four experiments

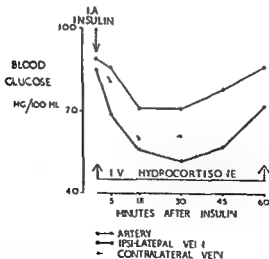


Fig. 4 Effect of an intra-arterial injection of insulin (1 unit) on arterial and venous blood glucose concentration during the intravenous infusion of hydrocortisone (0.6-1 mg/min.) mean of twelve experiments

responses to insulin (de Bodo and Altszuler<sup>6</sup>). The precise mechanism is not clear but there is much evidence that the adrenal steroids influence carbohydrate metabolism more by an increase in hepatic gluconeogenesis than by a decrease in the peripheral uptake of glucose (Altszuler *et al*<sup>7</sup> Froesch *et al*<sup>15,16</sup>). The present findings would support this view. The possibility that hydrocortisone may influence some phase of glucose utilization (Frawley<sup>14</sup> Henneman and Bunker<sup>20</sup> Hennes *et al*<sup>21</sup>) has not however been excluded by the present study.

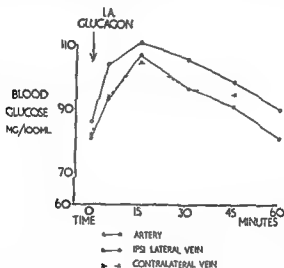


FIG. 5 Effect of an intra-arterial injection of glucagon (0.02 mg.) on arterial and venous blood glucose concentrations: mean of fourteen experiments.

#### PERIPHERAL EFFECTS OF GLUCAGON (Fig. 5)

Glucagon\* was given intra-arterially in a dose of 0.02 mg. Blood flow was measured and samples taken as before. Glucose concentration rose simultaneously and equally in the brachial artery and ante-cubital veins on either side. Peripheral blood flow did not change after the injection of glucagon. There was no selective rise or fall in the glucose level of venous blood draining the injected limb and no marked change in arterio-venous difference throughout. It was therefore not possible to obtain evidence of a direct peripheral action of glucagon in man. Since the general pattern of response

\* The glucagon used in these experiments was supplied by Eli Lilly & Co.

after arterial injection of the hormone was similar to that observed after intravenous administration any influence of glucagon alone on glucose uptake in the limbs would appear to be slight compared with its central effects

It has thus been possible to demonstrate in healthy adults a direct peripheral action of insulin and its fixation in forearm tissue. In this respect insulin differs from the other hormones studied for there was no evidence that hydrocortisone or glucagon had any direct influence on the peripheral uptake of glucose

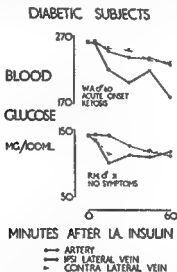


FIG. 6 Effect of an intra-arterial injection of insulin (1 units) on arterial and venous blood glucose concentration in two patients with diabetes

The technique was then used to assess the peripheral response to insulin in patients with disturbances of carbohydrate metabolism and altered sensitivity to the hormone. In view of the small numbers investigated at present and the difficulty of classifying patients with diabetes it is only possible to give selected examples of the response obtained

#### RESPONSES TO INTRA-ARTERIAL INSULIN IN DIABETIC SUBJECTS (Fig. 6)

Nineteen patients between the ages of 16 and 74 were investigated shortly after diagnosis and before treatment with insulin



The peripheral action of insulin was modified in obese diabetic patients with an insidious onset of the disease and with absence of ketosis — the so-called insulin-resistant diabetic (R M Fig 6) In these subjects the concentration of glucose fell equally in the artery and antecubital veins on either side There was no selective fall in the glucose level of venous blood from the injected limb There was thus an impairment of the direct peripheral action of insulin and possibly failure of immediate local fixation

In patients with a recent acute onset of the disease who were subject to ketosis the fall in glucose concentration was greater in blood taken from the ipsi-lateral antecubital vein than in samples from the contra-lateral vein or artery The high initial level of blood sugar did not modify the direct peripheral action of insulin and local fixation of the hormone was apparent in these subjects (W A Fig 6)

In all diabetic subjects glucose concentration fell progressively in arterial and contra-lateral venous samples Fasting levels were not restored after an hour as in healthy subjects

It is tempting to speculate on the relation between these responses and the different clinical manifestations of the disease or the various inhibitory factors described in diabetic subjects (Bornstein<sup>7</sup> Vallance-Owen<sup>27, 28</sup>) The small size of the present series however precludes further discussion

#### RESPONSE TO INTRA-ARTERIAL INSULIN IN CUSHING'S SYNDROME (Fig 7)

An altered response to intra-arterial insulin was observed in a patient with Cushing's syndrome secondary to adrenal hyperplasia The pattern of response was similar to that of obese diabetic subjects — an equal fall in arterial and venous glucose levels with no evidence of an immediate local action

Pituitary suppression was achieved by implanting radioactive yttrium Adrenal insufficiency and hypothyroidism developed after operation and maintenance prednisone and thyroid were given When the test was repeated 2 months after hypophysectomy the response to insulin was within normal limits There was a selective fall in venous glucose concentration in the injected limb and evidence of a local fixation of insulin

## RESPONSE TO INTRA-ARTERIAL INSULIN IN ACROMEGALIC SUBJECTS (Fig 8)

The effect of intra-arterial insulin was studied in six patients with acromegaly the disease was active in three cases and quiescent in the remaining three

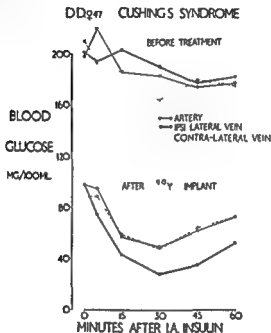


FIG. 7 Effect of an intra-arterial injection of insulin (a unit) on arterial and venous blood glucose concentrations in a patient with Cushing's syndrome before and after hypophysectomy

Fasting blood sugar was only raised in one patient but the peripheral action of insulin differed from normal in all six patients. Glucose concentration in the draining ipsilateral vein fell initially in five patients but control values were rapidly regained. The fall in the arterial and contra-lateral venous levels of glucose was slight by comparison with normal. Indeed in one patient there was no fall in glucose concentration in either vein or artery.

The altered response in patients with acromegaly was thus not

dependent on activity of the disease or on a raised level of circulating glucose. Furthermore the abnormality was distinct from that observed in obese diabetic patients.

### ACROMEGALIC SUBJECTS

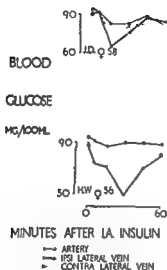


Fig. 8 Effect of an intra arterial injection of insulin (2 units) on arterial and venous blood glucose concentrations in two patients with acromegaly

### SUMMARY

1 The direct peripheral action of insulin and its fixation in healthy tissues has been demonstrated in fasting adults the uptake of glucose in the forearm was increased for at least 2 hours after an intra-arterial injection of insulin. This effect was not modified by the simultaneous intravenous infusion of hydrocortisone.

2 Hydrocortisone and glucagon had no direct influence on the peripheral uptake of glucose.

3 Peripheral responses to insulin were modified in obese diabetic subjects a similar response was observed in a patient with Cushing's syndrome.

4 A different abnormality in the peripheral response to insulin was observed in patients with acromegaly.

5 Further studies are required to determine the relation between these altered responses to insulin and the presence or absence of specific factors in conditions of endocrine imbalance.

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## SALICYLATE IN DIABETES MELLITUS

J REID and T D LIGHTBODY

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Interest in the action of salicylate in diabetes mellitus has been revived by the observation that aspirin given in the treatment of acute rheumatism also controlled the glycosuria and hyperglycaemia of a young diabetic. This action was confirmed in another group of diabetics and in addition to controlling glycosuria and hyperglycaemia aspirin was found to be capable of abolishing moderately severe ketosis.<sup>4</sup>

Disadvantages of the therapy are early development of nausea and vomiting in a few patients and milder but more frequent undesirable effects such as tinnitus and deafness though the latter tend to disappear with time. Nausea and vomiting are due to unsuspected overdosage with salicylate and are associated with very high serum salicylate levels. They are serious because they give rise to a starvation ketosis which is difficult to distinguish from diabetic ketosis and this confusion probably accounts for the abandonment of salicylate in diabetes long before the introduction of insulin. Fortunately nausea and vomiting need not arise if salicylate dosage is controlled by serum salicylate estimations in the early days of treatment so that none of the disadvantages really presents an insuperable problem to its use in diabetes. Indeed the fact that salicylate's long and continuous administration is not accompanied by risk of serious toxic effects such as agranulocytosis is in its favour for while this risk may be negligible in short term treatment it is not in a treatment which lasts for life. The practical value of aspirin in diabetes however cannot be decided until its potency has been compared with insulin the most effective anti-diabetic agent known.

We have investigated the insulin equivalence of aspirin in a group of fourteen patients whose insulin requirements ranged from 14 to 112 units each day. The patients were referred to us from the hospital diabetic clinic. Some were well controlled with insulin but a good proportion of difficult cases who were not well controlled included in the series. The complete practical range of

requirements has been covered and it may fairly be claimed that the group represents a cross-section of diabetics who require insulin.

Clinical particulars are shown in Fig. 1. Ages ranged from 33 to 74 years. Nine were females, five were males. The duration of the disease varied from 1 to 24 years, the carbohydrate intake from 104 to 295 g. daily and total calories from 1300 to 2700. The diets which the patients were accustomed to take as out-patients were continued throughout their whole stay in hospital that is for the

CLINICAL PARTICULARS

Patient	Age	Sex	Duration in years	Insulin Dose per week	Carb. Calcs. per week	Total Calcs. per week
H.L.	57	F	4	1 unit	104	300
R.M.	50	F	4	20 units	120	1400
A.C.	5	M		20 units	97	2400
S.M.	55	M	4	22 units	208	2000
E.L.	55	M	1	24 units	50	1900
S.H.	65	F	13	25 units	165	2000
J.C.	66	F	6	25 units	150	1700
S.H.	74	F	7	30	104	1400
C.M.P.	67	F	6	40	168	2000
H.M.	58	F	7	55 units	130	1800
G.L.	60	M	3	55 units	224	2300
M.E.	74	F	12	60	104	1500
R.C.	33	M	20	75 units	265	2700
C.M.R.	51	F	8	112 units	207	2000

FIG. 1

duration of the investigation except in one patient in whom a slight alteration was made during the insulin period. The only material change in insulin administration was to replace protamine zinc insulin with lente insulin in three patients so that all received the same type of insulin. Therapy was controlled by fasting blood sugar estimations and tests for glycosuria throughout the day. After an initial control period of at least 10 days during which the patient received their appropriate doses of insulin, aspirin therapy was started and insulin therapy continued. Aspirin was given in to attain maximum therapeutic serum salicylate.

# J REID AND T B LIGHTBODY

mg/100 ml Fasting blood sugar and urinary sugar estimate continued and insulin dosage was progressively reduced if warranted by the biochemical findings. The amount of insulin which could be replaced by maximum therapeutic doses of aspirin was determined for each patient.

Results are presented in Fig. 2 where it will be noticed that

INSULIN REQUIREMENT										SP 24	
PATIENT	O T			O U R A G			S A H		D I A B E T E S		
	C			E			E		N S I N		
	F A S T I N G			F A S T I N G			E Q U I V A L E N T		R E Q U I R E M E N T		
	mg/100ml	u	l	mg/100ml	u	l	mg/100ml	u	l	mg/100ml	u
1 M R	30	3		40			40				1
2 L	40	4		50			50				
C	67	30		72			60				20
2 J	50	24		154			57				1
3 W	85	38		210			90				28
J C	90	36		140			73				24
1 N	0	36		15			30				36
M C	04	8		40			65				3
5 M	200	20		272			26 900				0
6 M P	99	32		96 284			75 226				6
7 M M	30	3		55 240			3 216				4
8 L	114	12		61 271			40 390				1
9 C	299	76		73 262			7 216				10
10 M R	201	3		285			10				72

Fig. 2

possible to reduce insulin requirement of every patient after 2 to 3 weeks treatment with aspirin.

Eight patients with an initial requirement of 10 to 48 units of lente insulin were able to do without insulin altogether while aspirin was being given. Their fasting blood sugars at the end of the insulin and the aspirin periods were quite stable.

The next five patients had a modest reduction in insulin requirement of 10 to 20 units of lente insulin but the liability of the fasting



Fig 5 presents results in another fairly stable diabetic requiring the largest dose of lente insulin — 112 units per day. Insulin requirement in this case was reduced to 40 units per day and this probably is an understatement because fasting blood sugar and glycosuria were lower during aspirin therapy than they were in the control period with insulin alone. The insulin equivalence was therefore more than 72 units per day.

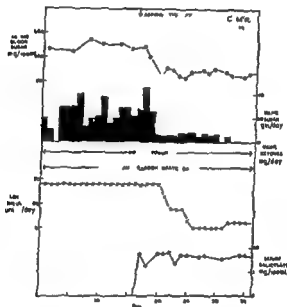


FIG 5 Findings in patient who was stabilized rather than controlled with 112 units of I Z S. The main effects of aspirin were a fall in fasting blood sugar to normal and diminution in glycosuria, accompanied by a reduction in insulin requirement to 40 units daily.

Finally Fig 6 illustrates what happens when the aspirin course precedes insulin administration. The high fasting blood sugar is quickly brought under control and there is negligible glycosuria during the period of aspirin therapy. After stopping aspirin the blood sugar takes just about as long to reach pre-aspirin level as it took to come down to normal. Thirty-six units of lente insulin per day were required to maintain the fasting blood sugar at a slightly higher control level.



These results place salicylate in the class of promising compounds for oral treatment of diabetes and justify carrying on investigations firstly to try to overcome the disadvantages of salicylate therapy already referred to and secondly to find out how it acts. The first of these investigations is under way. The second to find out how salicylate acts in diabetes will now briefly be considered.

In general there are two approaches to work of this kind — the biochemist's and the physician's. The biochemist tends to start with a simple biological preparation and work up to the whole organism. The research physician works in the opposite direction from the diseased patient to the normal and then to a simpler biological system. So far as diabetes is concerned the two approaches are mutually advantageous and this is exemplified by the recent findings of Manchester *et al.*<sup>2</sup> who noted that salicylate like insulin increased glucose uptake by isolated rat diaphragm. Unlike insulin however salicylate increases the potassium loss and diminishes the amino-acid incorporation in protein and it is not without interest that these biochemical observations on isolated muscle agree with the changes from salicylate we have observed in rheumatic patients.<sup>3</sup>

For our part it would perhaps not be so profitable to concentrate exclusively in looking for an explanation of salicylate's action in diabetes. Our interest in diabetes arose by chance while investigating the therapeutic action of the drug in rheumatic fever and in probing other possible therapeutic applications for it. This work was undertaken mainly to find out more about the nature of the disease processes. It would not however be appropriate now to do more than direct your attention to the amazing therapeutic versatility of this comparatively simple substance and then suggest in general terms how we might reduce it to understandable proportions.

Fig. 7 lists three disease states which are influenced by salicylate. They are rheumatic fever, diabetes mellitus and myxoedema. No apparent connection between the diseases is known. All three are also controlled by naturally produced hormones but each hormone benefits only one disease while salicylate affects them all though its range of action and potency varies. It is the drug of choice in rheumatic fever. It is not so potent as insulin in diabetes and it only affects some but not all of the abnormalities in myxoedema but its cholesterol-lowering effect in this disease is most pronounced and just as effective as thyroid hormones.<sup>1</sup> More recently it has been noted that salicylate lowers the cholesterol

level in other hypercholesterolaemic states including diabetes so that it would be well to keep this effect in mind when considering the problem of vascular degeneration — an important complication in diabetes

DISEASE	THERAPEUTIC AGENT	
RHEUMATIC FEVER	ASPIRIN	CORTISONE
DIABETES MELLITUS	ASPIRIN	INSULIN
MYXOEDEMA LOWERING OF CHOLESTEROL	ASPIRIN	THYROXINE

FIG 7

I am convinced that another approach to the problem of how salicylate acts in diabetes as well as in the other diseases is to continue the search for the fundamental biological action of the drug and to trace the whole series of effects which stem from it for it has been the more or less blind utilization of these individual effects for therapeutic purposes which in our present ignorance of their interrelations makes us wonder at and perhaps even doubt its remarkable therapeutic properties

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level in other hypercholesterolaemic states including diabetes so that it would be well to keep this effect in mind when considering the problem of vascular degeneration — an important complication in diabetes

DISEASE	THERAPEUTIC AGENT	
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# THIOL SUBSTANCES AND DIABETES MELLITUS

W J H BUTTERFIELD

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Sulphydryl substances are precursors in the synthesis of insulin and are also involved at various stages in intracellular carbohydrate metabolism. Lazarow<sup>13</sup> has reviewed the former question and discussed the steps whereby the  $\beta$  cells elaborate insulin from thio-amino acids such as cystine which constitutes over 10 per cent of the insulin molecule and discussed possible ways whereby diabetogenic substances for example alloxan interfere with these processes. I do not intend to go further into this here but shall consider in more detail the other aspect that is intracellular reactions which depend upon thiol-containing substances.

These reactions include the conversion of glyceraldehyde-3-phosphate into 1,3 diphosphoglyceric acid by triosephosphate dehydrogenase which enzyme includes glutathione, the cleavage of one carbon atom from pyruvate which involves a dithiol  $\alpha$ -lipoic acid, acetylation which involves Co-enzyme A and in the Krebs cycle first the conversion of  $\alpha$ -keto-glutarate to succinate also involving  $\alpha$  lipoic acid and Co-enzyme A and second the conversion of succinate to fumarate which involves succinic dehydrogenase. In addition Long *et al*<sup>14</sup> have published evidence that the phosphoglucomutase system may be thiol dependent.

My purpose is to review some pieces of evidence accumulated since 1950 suggesting that diabetes may be associated with an abnormality of thiol metabolism affecting some of these steps. The two points in carbohydrate metabolism on which I shall be focusing attention are first the action of insulin in facilitating the entry of glucose into cells and second the accumulation of pyruvate in the cells and its diffusion into the blood if it is not properly handled by being incorporated by intermediary metabolic processes and oxidized or stored as fat.

## I DIABETES AFTER INJURY

The first piece of evidence is based on abnormal carbohydrate metabolism after injury already alluded to briefly in the literature<sup>3,8</sup>



This study was concerned with burns a form of injury which permits the investigator roughly to assess the severity from the percentage of the body burned

Over a period of 18 months the burn service of the Medical College of Virginia in Richmond admitted 140 cases with burns of second or third degree of over 10 per cent body surface requiring resuscitation and skin grafting. These cases include patients of ages 2-81 years male and female white and coloured. All were resuscitated by a standard treatment and as soon as the initial phase of circulatory collapse was over (about 2 days) the patients received a high-protein high-caloric diet to offset the weight-loss so frequently observed after burns

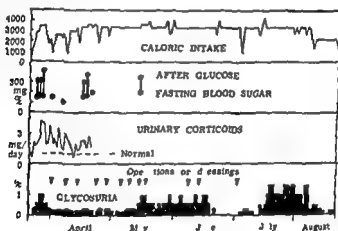


FIG. 1. An example of glycosuria following thermal injury

The present study began when I found by chance that one of these patients previously aglycosuric was passing sugar in her urine several days after being admitted to hospital. Thereafter arrangements were made to test 24-hour urine collections for glycosuria on all burns. At the end of 18 months of the total of 140 cases admitted 10 per cent had developed glycosuria some days after their injury. Since then several other cases have been observed in this country and colleagues in London, Birmingham, Edinburgh and various centres in the U.S. have also noted a similar condition.

Fig. 1 gives relevant information about an example case. The

patient suffered very deep flame burns of 19 per cent of his body surface was resuscitated in the usual way and feeding was started by the dietary service. The calories fed are indicated. The intensity of the glycosuria *which started a few days after injury* is also indicated. It will be observed that glycosuria persisted for about 4 months. It was aggravated by surgical procedures, dressings or operations. Some blood sugar estimations are recorded on this chart to indicate that the glycosuria was not renal in origin. Also included are estimates of the urinary formaldehydogenic corticoid excretion<sup>7</sup> which was elevated above normal levels. I shall return to this point presently.

Table I below summarizes the clinical findings in the cases of glycosuria following burns.

TABLE I  
SUMMARY OF 140 BURN CASES ADMITTED DURING 18 MONTHS  
FOURTEEN DEVELOPED THE DIABETIC STATE  
( ight m l    r x f males)

	R ge	Afean
Age	8.76	40
P cent burn	11.59	34
D y of o rt of glyco uria	1-9	6
Blood sug fasting	100-279	151
1 hour	199-450	153
A t ners	No e	
El t n of d betic state	Sh rte t in children Longest in the elde ly	

Of the possible causes for this diabetic state three came to mind early in the investigations. Since the first few cases had been resuscitated with a polysaccharide dextran one was concerned lest the diabetes be a toxic result of this substance. However it was presently traced in cases who did not receive any dextran. A second possibility was that the diet might be the cause of the diabetes. To ensure that the diet was not harmful *per se* eight volunteers were put to bed bandaged with burn dressings and given the same treatment for 10 days. None showed glycosuria all gained weight. Clearly the high diets\* alone were not the cause of the glycosuria.

\* Obviously the demands by the surgeons for feeding were completely contrary to the steps which the treatment of hyperglycemia required, but since these patients had to be kept in good nutritional condition for a long series of grafting operations, the surgical requirement tended to take precedence (see \* p. 613).

The other possibility was that these patients had hyperadrenocorticism. It was recognized that severe thermal injury is a potent stimulus to the adrenocortical axis<sup>2</sup> so adrenocortical function was assessed in nineteen burns and in eight volunteers. Table II summarizes the results for the first 5 days after injury in (i) the nine burns who subsequently developed glycosuria (ii) the ten burns who did not develop diabetes and (iii) the eight control subjects.

The results furnish good evidence that the group who developed diabetes temporarily had hypercorticism. Indeed in this respect one might regard them as similar to pregnant women in whom Hoet<sup>10</sup> and Jackson and Wolff<sup>11</sup> have observed abnormal carbohydrate metabolism.

TABLE II  
STUDIES OF ADRENOCORTICAL FUNCTION DURING FIRST 5 DAYS  
AFTER INJURY

	Nine burns with diabetes	Ten burns without diabetes	Controls (eight)
Mean age	37	33	34
Mean per cent burn	36	14	—
Mean corticoids mg/day	2.2	1.4	0.7
Mean eosinophils cu mm	6	30	105

*Carbohydrate metabolism within 3 weeks of injury* Turning to the special investigations of carbohydrate metabolism glucose (50 g glucose by mouth) and glucose-insulin (50 g glucose by mouth 5 units insulin i.v.) tests were performed. If tested within 3 weeks of injury these cases showed diminished glucose tolerance and absence of response to insulin in the glucose-insulin test (Fig. 2). Similar results are found in Cushing's disease or during ACTH and cortisone treatment.

The question arose what was the cause of the insulin resistance? Possibilities included insulin antagonists or some failure in intracellular carbohydrate metabolism. It was decided to test these two general possibilities by measuring the blood pyruvate levels in conjunction with the glucose and glucose-insulin tests. The results (Fig. 3) showed (i) abnormally high fasting levels and (ii) abnormally high rises during the glucose test. The pyruvate levels and rises

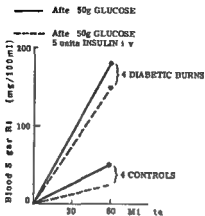


FIG 2 Mean rise of blood-sugar (mg/100 ml) during glucose and glucose-insulin in burns with hyperglycaemia and in controls

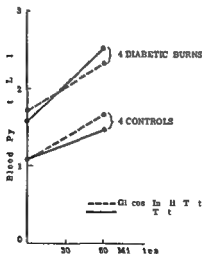


FIG 3 Mean pyruvate (mg/100 g blood) in glucose and glucose-insulin tests in burns with hyperglycaemia and in controls

were much greater than observed in non-diabetic burns even of equal severity or extent or in controls or in insulin-dependent diabetes<sup>3</sup>

It was therefore difficult to reconcile these findings with any explanation for insulin resistance based on insulin antagonists during the glucose and glucose-insulin tests glucose must have penetrated the cells and been broken down to pyruvate which then diffused back into the blood stream. The accumulation of the pyruvate observed was accepted as evidence that there was some alteration in intracellular carbohydrate metabolism in some tissue or tissues.

The notion that the accumulations of glucose and pyruvate in the blood could be due to the excessive diet had already been dispelled but the contrast between the controls who tended to gain weight on high calorific diets and the diabetic burns who tended to lose weight and go into negative nitrogen balance offered a clue. The diabetic burns exhibited a metabolic block presumably at or below the level of pyruvate oxidation which interfered with the usual relationship between carbohydrate consumption on the one hand and fat deposition and protein sparing on the other.

About that time Conn and his colleagues reported that intravenous glutathione had lowered the blood sugar of a female volunteer made diabetic by ACTH injections<sup>6</sup>. The pyruvate oxidation system being dependent upon two thiol substances lipoic acid and Co-enzyme A it was decided to explore the effects of BAL on the glucose tolerance in (i) the diabetic burned patients (ii) patients who were burned but not diabetic and (iii) in non-diabetic controls. 150 mg of BAL were given i.m. shortly before the start of the usual glucose tolerance test. In the latter two groups BAL was without effect but in some of the diabetic burns the thiol appeared to improve glucose tolerance (Fig. 4). Furthermore the rise in blood pyruvate was smaller in the BAL-glucose test than when glucose was given alone.

It should be stressed that these results obtained only in patients who were tested within 3 weeks of their injury after that time the diabetic burns became more insulin-sensitive less responsive to BAL alone but did respond when treated with both insulin and BAL<sup>3</sup>.

The tentative conclusion was that an abnormality of SH metabolism operating at the level of pyruvate oxidation and most pronounced soon after the injury would explain the findings. It seemed unlikely that the patients could be suffering from a deficiency of

the monothiol Co-enzyme A since they were able to break down and presumably utilize the body depot fat. On the other hand the results with the dithiol BAL suggested that the biochemical lesion was associated with the inability of pyruvate to enter intermediary metabolism a step which involves the natural dithiol  $\alpha$  lipoic acid.

One other point remained for examination. Was a similar biochemical lesion detectable in non diabetic burns made glycosuric by ACTH? Hyperpyruvataemia has been reported after ACTH and cortisone by several investigators<sup>9,10</sup>. In the course of a skin-grafting trial ACTH was given to a burn. Hyperpyruvataemia and sensitivity to BAL were found<sup>4</sup>.

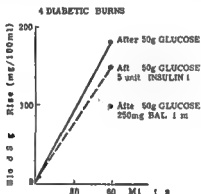


FIG 4 Mean rises of blood-sugar levels in glucose-glucose-insulin and glucose-BAL tolerance tests

The order of the day at this symposium is speculation. Setting aside the fact that these investigations were carried out in burned patients where many pathological factors operate — infection, tissue necrosis and so on — and assuming that the action of BAL is on SH metabolism and not due to any chelating effect<sup>16</sup> it is tempting to suggest that stress situations may be associated with a change in SH metabolism<sup>1</sup>. If this affected all tissues for a few weeks it would explain the insulin resistance and the hypoglycaemic effect of BAL. Later on this metabolic block might be expected to leave its mark on the function of the  $\beta$ -cells themselves interfering with their utilization of glucose and therefore presumably with their production of insulin. This would explain the subsequent insulin sensitivity.

Various aspects of the foregoing investigation have been studied in established medical as opposed to traumatic diabetes as follows

## 2 EFFECT OF THIOL SUBSTANCES IN INSULIN-RESISTANT DIABETES

One of the features of the diabetes described above after burns was insensitivity to insulin in the glucose-insulin test. Two diabetic cases showing marked insulin resistance have therefore been studied for the effects of thiol substances \*

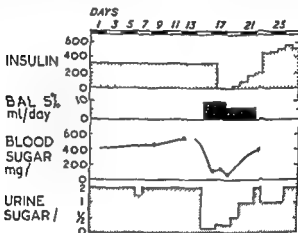


FIG. 5 Effect of BAL on blood sugar and glycosuria in a 37 year-old diabetic

The first was a 37-year-old plump coloured woman a severe diabetic of many years standing who had been treated with gradually increasing doses of insulin without satisfactory control. She was suffering from diabetic neuritis and Kimmelstiel-Wilson's syndrome. She showed intense glycosuria but no acetonaemia, her blood sugar rising up to 540 mg per cent (Fig. 5). After a suitable control period on a fixed diet and 320 units of insulin/day BAL (150 mg in oil 1 m three times a day) was started in addition to the insulin. Next day her urine showed only a trace of glucose and her fasting blood sugar was 118 mg per cent. The following day insulin was withheld but BAL continued. There was a trace of sugar in the urine, the fasting blood sugar was 140 mg per cent rising to

\* These are cases 1 and 2 in \* Figs. 5 and 6 from that publication.

190 mg per cent 1 hour after 50 g glucose by mouth indicating greatly enhanced glucose tolerance

On the fourth day the fasting blood sugar was 81 mg per cent and there was very little glycosuria

When BAL was stopped intense glycosuria reappeared immediately and the blood sugar rose to 400 mg per cent despite re-instituting high doses of insulin

The second case was similar. A 36-year-old white woman a severe obese diabetic of 15 years standing with diabetic neuritis and Kimmelstiel-Wilson's syndrome was uncontrolled by large doses of insulin varying up to 2000 units/day she showed no acetonuria. She was admitted to hospital for study. Examination of her urine for  $\alpha$  keto acids by Dr Brian McArdle showed high values of both pyruvate and  $\alpha$  ketoglutarate (see Table III) which may be inter-

TABLE III  
MEAN URINARY EXCRETIONS OF PYRUVATE AND  
 $\alpha$  KETOGLUTARATE

	P t t	C trols	P
Pyruv t mg	23	69	< 001
ketogl t 10 mg	63.5	35.4	< 001

preted as evidence for impaired metabolism of these intermediary compounds both of which require lipoic acid for their metabolism

After a control period on a fixed diet of 1600 calories and 480 units of insulin/day BAL (200 mg t d s) was given for 3 days (see Fig. 6). A prompt reduction in blood sugar and urine sugar occurred. When the BAL was withdrawn the blood and urine sugars rose to previous values. After 5 days BAL was again administered (200 mg four times/day). There was again a fall in blood sugar and glycosuria.

This case was studied intensively over a period of 3 months. The response to various doses of BAL for 3 or more days was studied the patient being maintained on diets of 1600 and 800 calories a day. The mean daily glycosuria on the various experimental conditions different diet and altered doses of BAL are shown in Fig. 7. It will be seen that the effect of BAL was progressively greater as the dose was increased.



Various aspects of the foregoing investigation have been studied in established medical as opposed to traumatic diabetes as follows

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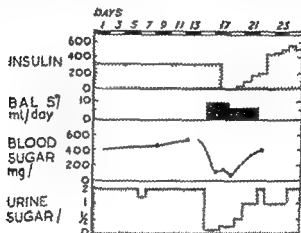


FIG. 5. Effect of BAL on blood sugar and glycosuria in a 37 year-old

The first was a 37-year-old plump coloured woman diabetic of many years standing who had been "gradually increasing doses of insulin without satisfaction. She was suffering from diabetic neuritis and Kimmeri syndrome. She showed intense glycosuria but no actual blood sugar rising up to 540 mg per cent (Fig. 5). A control period on a fixed diet and 3.0 units of insulin (150 mg in oil) in three times a day) was started on the insulin. Next day her urine showed only a trace of sugar. Her fasting blood sugar was 118 mg per cent. The insulin was withheld but BAL continued. There was a trace of sugar in the urine. The fasting blood sugar was 140 mg

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190 mg per cent 1 hour after 50 g glucose by mouth greatly enhanced glucose tolerance

On the fourth day the fasting blood sugar was 81 and there was very little glycosuria

When BAL was stopped intense glycosuria immediately and the blood sugar rose to 400 mg per cent re-instituting high doses of insulin

The second case was similar. A 36-year-old white severe obese diabetic of 15 years standing with diabetic Kimmelstiel-Wilson's syndrome was uncontrolled by large doses of insulin varying up to 1,000 units/day. She showed no acidosis. She was admitted to hospital for study. Examination of blood for  $\alpha$ -keto acids by Dr Brian McArdle showed high values for pyruvate and  $\alpha$ -ketoglutarate (see Table III) which may be

TABLE III  
MEAN URINARY EXCRETIONS OF PYRUVATE AND  
 $\alpha$ -KETOGLOUTARATE

	Patient	Control	P
Pyruvate mg	33	69	< .001
$\alpha$ -ketoglutarate mg	63.5	35.4	< .001

interpreted as evidence for impaired metabolism of these intermediate compounds both of which require liponic acid for their metabolism

After a control period on a fixed diet of 1600 calories and 280 units of insulin/day BAL (200 mg t.i.d.) was given for 3 days (see Fig. 6). A prompt reduction in blood sugar and urine sugar occurred. When the BAL was withdrawn the blood and urine sugars rose to previous values. After 5 days BAL was again administered (200 mg four times/day). There was again a fall in blood sugar and glycosuria.

This case was studied intensively over a period of 3 months. The response to various doses of BAL for 3 or more days was studied the patient being maintained on diets of 1600 and 800 calories a day. The mean daily glycosuria on the various experimental conditions, different diet and altered doses of BAL, are shown in Fig. 7. It will be seen that the effect of BAL was progressively greater as the dose was increased.

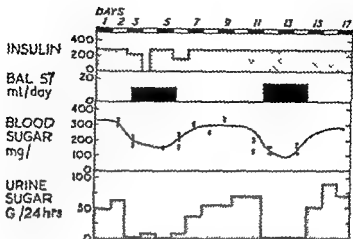
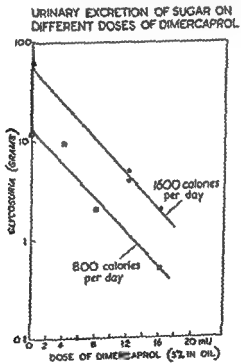


FIG 6 Effect of BAL on blood sugar and glycosuria in a 36-year-old diabetic



Effect of BAL on 24 hour glycosuria (g) diet 1600 cal/

About 12 months later the opportunity arose to study the effect of another SH compound in this patient. A small supply of mercaptoethylamine salicylate was made available to us\*. Ignorant of the sort of dose regime to use we started with 100 mg† capsules four times a day given when the patient was out of control on 1000 units insulin/day (Fig 8). After a delay of 3 days during which no response was detected the patient unexpectedly became normoglycaemic and the thiol was stopped. Blood-sugar levels were in

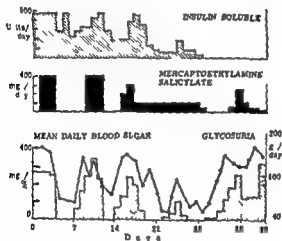


FIG 8 Effects of mercaptoethylamine salicylate in an insulin-resistant diabetic

the normal range for 2 days after which the blood and urine sugar values rose. Mercaptoethylamine was given again. After a delay similar to the first latent period the blood sugar fell again rising later on the withdrawal of the thiol. Attempts were then made to achieve satisfactory control of the diabetes by adjustments to the doses of insulin and mercaptoethylamine but without success. During the subsequent period of trial with mercaptoethylamine the patient's course swung slowly from hyper- to hypo-glycaemia

\* In knowledge of kind co-operation of Mr E. M. Barrett of Smith & Nephew in supplying the compound.

† Equivalent to less than 1 gr. as a patient's system did not suffice the glycosuria in this patient.

from week to week the excursions getting gradually larger. On occasions she passed over 250 g of glucose/day in the urine only to pass several days later into a period of severe hypoglycaemia the control of which once required 1100 g of carbohydrate over a period of 24 hours.

Many months later this patient was controlled by sulphonylurea treatment suggesting that she was capable of producing her own insulin.

One was tempted to regard the initial aspects of the mercaptoethylamine study as evidence that the monothiol which showed a delayed action in contrast to the dithiol had required metabolic alteration to exert its effect. However a full explanation of the extraordinary course subsequently shown by this patient is not possible at present. The powerful metabolic action of the thiol could be due to some relationship between diabetes and thiol metabolism or to an action releasing insulin from binding substances in the blood. Trials of mercaptoethylamine in other diabetics have not been encouraging.

### 3 HYPERPYRUVATAEMIA IN ESTABLISHED DIABETES

Another feature of the initiation of a diabetic state in the burned patients was the occurrence of hyperpyruvataemia after glucose and insulin. It was decided to test established diabetes in the same way seeking evidence for a similar hyperpyruvataemia which might be a residuum of the initial metabolic disturbance.

To date over fifty diabetics have been investigated. Abnormally high pyruvate levels have been found in the venous blood of about a third and especially high values occur in the severe poorly controlled (brittle) diabetics.

These results were presented recently at the International Diabetic Congress by Dr Kelsey Fry, Professor R. H. S. Thompson and myself<sup>5</sup> and I will only summarize them here.

We find that brittle diabetics have a pronounced rise in blood pyruvate during the first hour of a glucose-insulin tolerance test (see Table IV).

Normal subjects and most diabetics show a modest rise of 0.3 and 0.4 mg per cent respectively during this period whereas values of up to 3.4 mg per cent with a mean rise of 1.7 mg per cent have been recorded in the brittle cases, values which are comparable to those found in the diabetic burns.

However we are now in a position to offer a little additional information about these patients. Measurements of A-V differences across the forearm muscles in conjunction with blood-flow estimations make it possible to assess the peripheral utilization of pyruvate during a glucose-insulin test. Preliminary data show that the arterial level of pyruvate may be considerably in excess of the venous level indicating that there can be peripheral utilization of pyruvate indeed during this phase there is negligible uptake of glucose and it might be argued that the tissues have a slight compensatory mechanism by introducing carbohydrate into the cells as diffusable 3-carbon pyruvate rather than as 6-carbon glucose<sup>1</sup>

TABLE IV

MEAN RISE OF PYRUVATE DURING GLUCOSE-INSULIN TOLERANCE TEST (MG) (100 G BLOOD)

	<i>Control</i>	<i>Diabetics (brittle)</i>	<i>Diabetics (ther)</i>
Rise at 60 min	0.3	1.7	0.4

The main implications of the findings of high arterial pyruvate levels are firstly that the pyruvate is probably elaborated in the splanchnic area and secondly that in our previous studies peripheral uptake by muscles could have masked the release of larger quantities of pyruvate by the splanchnic area than was suspected during the glucose insulin test. Thus faulty pyruvate metabolism may be present in a larger proportion of the diabetic population than is at present recognized and the incidence of hyperpyruvataemia on the arterial side must be sought.

Whether the hyperpyruvataemia found in these cases of established diabetes reflect a similar abnormality to that obtaining in the temporarily diabetic burned patients cannot be answered. However we have reported that like the diabetic burned patients their glucose tolerance appears to be improved by BAL given for 3-4 days<sup>4</sup>. These findings are in harmony with those of Mozo<sup>15</sup> and Asper and his colleagues<sup>16</sup> who have all reported improvement of the diabetic state during treatment with BAL.

In view of the previous evidence we have been tempted to speculate on the possibility that these patients suffer from some

disorder of thiol metabolism perhaps a relative deficiency of  $\alpha$ -lipoic acid in the splanchnic region which was corrected temporarily by the injections of BAL<sup>14</sup>

#### 4. CONTROL OF TWO YOUNG UNSTABLE DIABETICS WITH BAL ON AN OUT-PATIENT BASIS

To examine the role of BAL in therapy two young unstable brittle diabetics who responded to BAL for a few days in hospital were treated in conjunction with Drs C Hardwick and C L Joiner on an out-patient basis

The first case was a 26-year-old woman who had been diabetic since the age of 17. Her insulin requirements had gradually risen over the years and she was uncontrolled on 100 units insulin/day when the present study began. Her blood pyruvates were elevated after glucose and insulin. When she was tested with BAL in hospital her glycosuria diminished over the last day on the diethiol and her pyruvate levels during the glucose-insulin test fell. On the withdrawal of BAL her diabetes returned to the previous uncontrolled state. She returned home and was kept under close surveillance as an out-patient. Her urine was tested before meals and recorded four times a day for 3 months: the majority of these tests showed glycosuria (Table V). Insulin injections were then augmented with

TABLE V

EFFECT OF BAL ON GLYCOSURIA DURING OUT-PATIENT STUDY ON 6-YEAR-OLD DIABETIC UNCONTROLLED ON 100 UNITS INSULIN/DAY

Test result	Frequency on 100 units insulin/day (3 months)	Frequency on 100 units insulin/day and BAL (3 months)	Statistical analysis (2 x 2 contingency)
> 2 per cent	21 per cent	0.5 per cent	$P < .001$
2 per cent	23 per cent	0.5 per cent	$P < .001$
1 per cent	40 per cent	7 per cent	$P < .001$
$\frac{1}{2}$ per cent	10 per cent	per cent	$P < .001$
0 per cent	6 per cent	70 per cent	$P < .001$

BAL 200 mg as 5 per cent solution in oil i.m. three times/week urine testing continuing. Over the first month there was diminution of the glycosuria which was maintained. By the end of 2 months the majority of urine tests were negative for sugar (Table V). During this period the patient had a few episodes of moderate

hypoglycaemia she also gained weight. Unfortunately after 3 months albuminuria was detected. This was attributed to the BAL. Renal biopsy was performed: no microscopic abnormality was detected. BAL was then stopped. The albuminuria cleared up within a week and the diabetic control was gradually lost. A subsequent course of BAL was also associated with improved control but albuminuria recurred. It was deemed inadvisable to continue this therapy further.

Similar findings were made in a 20-year-old male patient with unstable diabetes who also had high levels of pyruvate especially in arterial samples during the glucose-insulin test. His out-patient regime before BAL included 130 units insulin/day. During the control period before BAL the majority of the urine tests performed before meals indicated heavy glycosuria.

BAL was given 100 mg 5 per cent in oil three times/week. There was a clear-cut reduction in the number of glycosuria urine samples over the subsequent 2 months of treatment. Statistical analysis again indicated that the reduction of incidence of glycosuria was statistically significant. The patient suffered several hypoglycaemic attacks and his insulin dosage had to be reduced to 100 units/day. However he too developed albuminuria and the thiol had to be discontinued. Withdrawal of BAL was followed by a reversion to the previous uncontrolled diabetic state without albuminuria. We attributed the albuminuria to the chelating effects of BAL on trace metals involved as enzymatic co-factors in the kidneys. As the patients gained weight on BAL its action was not due to interference with glucose absorption. Nevertheless we must conclude that BAL offers no prospect as a therapeutic agent for the long term control of unstable diabetes.

### CONCLUSION

In each of the four investigations reviewed here the facts may be reconciled with a general hypothesis that diabetes may frequently be associated with an abnormality of thiol metabolism. However such a reconciliation does not mean that the explanation offered is necessarily correct. It is salutary to recall Hamlet's exhortation — There are more things in heaven and earth, Horatio, than are dreamt of in your philosophy.



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# A COMPARISON OF THE MECHANISM OF ACTION OF INSULIN WITH THAT OF THE HYPOGLYCAEMIC SUBSTANCES

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Any French accent which you may detect comes from the laboratory in Montpellier since I am substituting for Professor Loubatières

When Professor Young asked me late last night after several assorted alcoholic beverages to do this job I did not have the strength of character to resist. He expected that I should have (and was disappointed that I did not have) any slides with me.

Now to the subject matter itself. The blood-sugar lowering effects of certain sulphonamide derivatives was noticed sporadically some few years before the fundamental discovery by Jambon and Loubatières. No attention was paid to it because it was considered to be a toxic side effect. But as you well know Jambon noticed the intense hypoglycaemia after the administration of isopropylthio-diazole in 1942. Immediately thereafter Professor Loubatières at Montpellier undertook the physiological and pharmacological study of this and other closely related compounds and he did a magnificent job in the 4 years between 1942 and 1946. We must cast our glance back to those years and remember that a war was raging at that time. We must consider the difficulties under which these papers were published because they were published in 1946. They received little notice except from a few isolated individuals namely La Barre in Belgium and Chen in the United States. A few years later the hypoglycaemic property of another sulphonamide (known as BZ 55) was discovered in Germany and soon after the effects of its methylated derivative — tolbutamide. The pharmacological work in Germany took at first an entirely different direction from that of Loubatières. The clinical work began almost immediately and actually we had few pharmacological data about these compounds at that time after several hundreds of patients had already been

treated. I should like to remind you that by 1946 Loubatieres had established for IPTD (isopropylthio-diazole) certain experimental facts which have since then been abundantly confirmed also for the newer sulphonylureas.

The experimental animals were dogs and rabbits. In the normal dog one could lower the blood sugar with sulphonylureas. If the animal's pancreas were completely removed the acute administration of the sulphonylureas by mouth or by any other route no longer produced lowering of the blood-sugar level. If the pancreas were removed only partially, the blood sugar would fall. Loubatieres therefore called these substances pancreatotropic and considered that they tended to liberate insulin from the  $\beta$  cells. About one-sixth of the pancreas of the dog was needed to produce a *brisk* hypoglycaemia. It was also established that nerve pathways were not concerned in the production of hypoglycaemia (The spinal cord, the splanchnic nerves and the vagi). The endocrine glands other than the pancreas were not necessary for the demonstration of the IPTD hypoglycaemia. The glands were excised one at a time in the same animal. Neither thyroidectomy, castration, adrenalectomy nor hypophysectomy singly or jointly interfere with the blood-sugar lowering effect. The final operation, i.e. of the pancreas, abolished the action.

The effects of these drugs were then tested in alloxanized diabetes. Alloxan diabetes of course is a variable state since one may have varying degrees of destruction of the  $\beta$  cells. The  $\alpha$  cells are maintained in normal histological state in alloxan diabetes. In severely alloxanized rabbit these drugs did not lower the blood sugar. However, if there was only a partial alloxan diabetes the blood sugar was lowered despite the fact that the  $\alpha$  cells were intact. This is important because some time later studies of von Fernet and others implicated a depression of the  $\alpha$  cells, therefore presumably a depression of glucagon manufacture and secretion as the reason for the hypoglycaemia. This view has no real support even by its original proponents.

Loubatieres's early work showed therefore that the prerequisite for the acute hypoglycaemic action of the sulphonylureas is the presence of a sufficient number of normal  $\beta$  cells in the pancreas. The  $\alpha$  cells do not play any significant role in this.

The success of the clinical testing of the sulphonylureas in the last few years has spurred on a large volume of research

intimate nature of the mechanisms of action. We shall now present some of the salient features of the general results in this field. Let us examine first the possibility of direct action on the peripheral tissues from the results obtained in experiments done on liverless and eviscerated animals and on isolated muscle fat or such other tissues which are normally insulin sensitive with a view to determining whether the sulphonylureas imitate in any way the action of insulin. With very few exceptions the consensus of opinion at this moment is that by themselves the sulphonylureas do not increase the utilization of glucose, do not change the oxygen consumption or  $\text{CO}_2$  production and do not lead to a distribution of the non-utilizable insulin sensitive sugars. They therefore in no way imitate the action of insulin.

From the previously cited evidence of Loubatieres which more recently was confirmed and extended by Professor Houssay it is evident that sulphonylurea hypoglycaemia is not dependent on the inhibition of contra-insulin factors in the pituitary-adrenal etc.

The importance of the  $\beta$  cell was substantiated in man. Diabetes developing after surgical pancreatectomy was not influenced by these drugs. Juvenile diabetes in which Wrenshall has shown that the pancreas possesses a very low insulin content cannot be treated with these drugs. However adult onset diabetes of the obese non-ketotic variety in which pancreatic insulin content tended more towards the normal values can be treated both acutely and chronically by sulphonylureas showing the importance of the  $\beta$  cell in the response of human diabetes mellitus.

Houssay, Caren and others since have observed that under certain conditions in the depancreatized animal when the dose of insulin is small and the dose of sulphonamide is rather large one can see a potentiating effect in the absence of  $\beta$  cells but in the presence of exogenous insulin. These findings disturb the unitary hypothesis that the action of sulphonylureas is only on the  $\beta$  cell.

If insulin is indeed the mediating agent in the action of the sulphonylureas either by stimulating the  $\beta$  cells to secrete insulin or by potentiating it peripherally or both then one should see in the animal or man given the sulphonamides the same metabolic effects one gets after insulin. Many of these have been looked for and the literature on the subject is most confusing and contradictory. A rise in pyruvate occurs after insulin and glucose administration. After tolbutamide despite the lowering of the blood sugar the rises in

pyruvate were not obtained by some workers and were seen by others. An increase in A-V differences of glucose in the peripheral limbs was obtained by some and not seen by others. I think Dr Butterfield is numbered among those who did obtain them with tolbutamide if I am correct.

It was supposed that if a certain amount of insulin lowered the blood sugar by 40 mg per cent and if one gave tolbutamide to lower the blood sugar an equal 40 mg per cent one should expect exactly the same results in all other blood constituents. If indeed the tolbutamide acts by releasing insulin from the pancreas then it would release it not in 10-unit amounts as if from a syringe but rather in very very small amounts at a constant rate over a certain period of time. Also the liberated insulin would enter the portal system and not a peripheral vein. Thus the insulin could act on the liver before it came to the periphery or a large portion of the insulin could be destroyed by insulinase in the liver itself.

One of the earliest workers to cast doubt on the neat picture of a pure insulin effect of the sulphonylureas was Dr Renold who did a simple experiment. It is always the simple experiments which are bothersome. He showed that when one gives fructose to diabetic the blood glucose rises as a result of transformation in liver. When tolbutamide was given the glucose rise was pointing to an effect perhaps an inhibition of liver sugar. We therefore come full tilt up against the problem of the of the sulphonamides not in the periphery but upon the liver. This problem has two facets and these two facets can best be characterized by calling them the evident aspects of the phrenia of the subject. If one holds that the sulphonamides stimulate the  $\beta$  cell that insulin is then released via the portal system reaches the liver and if the sulphonamides have no other action than this then there arises the contradiction that the output of sugar by the liver seems to be inhibited by the sulphonamides but not by the direct infusion of insulin.

Weinhouse *et al* using  $^{14}\text{C}$  glucose find that the output of glucose by the liver is inhibited by both the sulphonamides and insulin. However Schambye's data are the same as those of the group namely that the sulphonamides inhibit sugar output by the liver but that insulin does not.

The sulphonylureas do not lower the blood sugar of an animal from whom both the liver and pancreas have been removed.

ever if the pancreas is left in the abdomen (by porto-caval anastomosis) when the liver is removed and the blood-sugar level is maintained by the constant infusion of glucose the sulphonylureas will produce hypoglycaemia. Thus the liver is not an indispensable organ for the hypoglycaemic effect. That does not mean however that when the liver is in that the tolbutamide does not have a direct or indirect action on the liver as well.

The evidence for the liberation of insulin from the pancreas by the sulphonylureas is quite good especially when one adds to that the work of Pfeiffer (done with the Harvard group) in which the injection of tolbutamide increased the plasma insulin level especially in the portal system. This view does not explain the potentiating action in the depancreatized animal. A few years ago when we were dealing with these confusing aspects Dr KrahI suggested that insulin may combine with a tissue component and thus remain inactive and that the sulphonylureas split this complex. They would then liberate insulin from the complex and make it available. Such an action may occur in the  $\beta$  cell but also in the peripheral tissues which would account for the pancreatic as well as the potentiating effect.

Since the title included other oral hypoglycaemic agents I should like to comment shortly about another type that is at present under clinical and experimental trial namely DBI. The biguanides chemical relatives of synthalin seem to act in the absence of the  $\beta$  cell on peripheral tissues e.g. on muscle *in vitro*. They stimulate like Dr Randle's dinitrophenol and other depressants of cell respiration the uptake of sugar by the cell. The action resembles the state of partial anaerobiosis and just as in anaerobiosis the lactic acid in the blood increases tissue glycogen falls and some of the oxidative systems of the cytochrome series are inhibited. We are dealing with an inhibition of oxidative metabolism and a consequent greater anaerobic utilization of glucose under these circumstances.

It is contended on the basis of A-V differences done over a short time period that as a result of tolbutamide action there is no increased utilization of carbohydrate. It is however impossible to maintain such a conclusion for the following reason. Suppose that a 50-year-old adult obese diabetic is excreting 30 to 50 g of sugar in the urine and requires for control about 30 units of insulin per day and eats a diet of 1500 to 1800 calories per day including 450 g of carbohydrate. He is then placed on tolbutamide and maintained

on it for 1 or 2 years. At the end of that period he is well and healthy, has been working satisfactorily, has no glycosuria, is not emaciated and his fat depots are normal. It would seem that all of the dietary carbohydrate ( $365 \times 250$  g per year) must have been utilized in a perfectly normal manner in such an individual.

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#### DISCUSSION

LONG I was particularly interested in Dr Ginsburg's failure to demonstrate any peripheral effect of hydrocortisone even though it was given in very large amounts over a relatively short period of time. It has been our experience using animal preparations — eviscerated nephrectomized rats in fact — that it is not possible to demonstrate any effect on either the utilization of glucose by these animals or any modification of the response to insulin when large amounts of hydrocortisone are given. This of course contrasts very sharply with the ready demonstration of the effect of the same hormone on the carbohydrate metabolism of the liver and I think emphasizes what I hinted at yesterday that in all probability the main effect of the adrenal steroids is directed towards the carbohydrate metabolism in the liver and that any effects that may occur in the peripheral tissue are probably secondary to this. For example in the so called steroid diabetes it can be shown that first effects which follow the administration of hydrocortisone are a great accumulation of glycogen in the liver. This is followed at a much later period by an increase in the muscle glycogen undoubtedly due to the fact that at the time when the liver glycogen has risen to these high levels the blood sugar has also risen. One other point in relation to the adrenal cortical hormones and this question of the demonstration of glycogen synthesis particularly in liver slices is that it has been our experience in adrenalectomized animals that the ability to retain or perhaps form glycogen in the liver from a variety of precursors is very considerably diminished in the absence of the adrenal corticosteroids. I have been wondering whether in many experiments where attempts have been made to demonstrate liver glycogen synthesis as for example in slices by the addition of insulin this will be possible unless cognizance is taken of the important role played by the adrenal steroids in the formation or perhaps we should even say the retention of glycogen in the liver.

STEWART Following on Dr Wolff's paper I would like to make one or two observations

Firstly Madison and Unger recently described the effects of administering insulin endoportally and peripherally and found the distribution of the insulin was markedly different. By the endoportal route 51 per cent was found in the liver and .7 per cent in muscle and by the peripheral route the ratio changed and 56 per cent was found in the muscle and only .7 per cent in the liver. The A-V glucose difference was smaller after endoportally administered insulin than after peripherally administered insulin. We believe that we are stimulating the release of endogenous insulin and therefore some of the differences in our experiments within a group may possibly be explained by the way in which the endogenously released insulin is distributed throughout the body of each subject.

Another point is the question of the diabetics who are non ketonic. I remember Professor Young saying a short while ago that he had seen some dogs which were rendered diabetic by pancreatectomy that the administration of a minute amount of insulin removed the evidence of ketosis without affecting the blood sugar level and that too maybe has some bearing on these differences.

Fig 1 shows the effect of administering both carbutamide and tolbutamide to groups of guinea pigs and determining the maximum fall in blood sugar within these groups. The third group which received insulin is also shown. The main point I want to bring out is that with the sulphonylureas there is a dose beyond which as it is increased the amount of hypoglycaemia gets less and although these doses in animals are much larger on a body weight basis than those given to man. Dr Wolff and I and others have shown that in man this sort of effect can take place with much smaller doses. It may be due to a stimulation of the adrenals as Bander has shown.

Fig 2 shows the effects of administering glucose and tolbutamide to two groups of rabbits. The groups on the right are rabbits which have at one time been rendered alloxan diabetic and then allowed to recover in so far as their blood sugar level came back to the normo-glycaemic range. When tolbutamide is administered to these rabbits there is during the first 2 hours no evidence of hypoglycaemia compared with the response in the normal rabbits. When glucose is subsequently given the tolerance of these animals which have shown a remission of the alloxan diabetes is less than that of the normal animals. Interestingly enough the rabbits which have shown this remission of alloxan diabetes have histologically normal  $\beta$  cells of the pancreas according to my colleague Dr Udall and also their insulin content can be shown to be normal by bio-assay. So it may be that the suggestion Dr Wolff made that it



sulphonylureas may act on some specific mechanism in the pancreas to cause the release of insulin may be an important factor in their mode of action.

HOET In relation with the mode of action of hypoglycaemic sulphonylurea, I wish to draw the attention to experiments done by de Meyer and M Isaac-Mathy about the teratogenic action of BZ 55

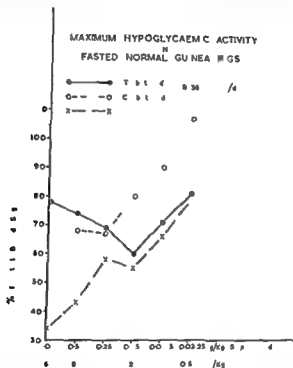


FIG 1 The effect of dose on the maximum hypoglycaemic response of fasted normal guinea-pigs to tolbutamide tolbutamide and insulin (tolbutamides administered orally insulin subcutaneously)

When a single peroral dose of 500 mgms is given between the ninth to the eleventh day of pregnancy the rat delivers newborn who are malformed oedematous and in a vast majority anophthalmic. This is absolutely different from the pictures of some bone abnormalities seen after profound and repeated hypoglycaemic convulsions. With BZ 55 the pregnant rat has no symptoms but the offspring has lesions of the eyes and the optic nerve. Another sulphonamide — not hypoglycaemic

did not result in any teratogenic effect. As long as this teratogenic effect is not clearly understood and as long as we are not absolutely certain that side effects on the foetus are not harmful it seems advisable not to give BZ 55 to pregnant women not even when the carbohydrate disturbance is mild (de Meyer et Isaac Mathy. A propos de l'action teratogène d'un sulfamide hypoglycémiant (N sulfanil N nulylée — BZ 55) *Les ann d'Endocr* 1958 XIX 1 168-7-).

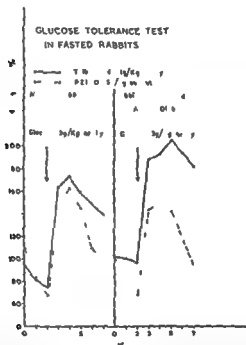


FIG. 2. The tolerance of normal rabbits and of rabbits which have recovered from alloxan diabetes to glucose 2 hours after they have received either tolbutamide or protamine zinc insulin (PZI).

YOUNG: I am sure you will all agree after hearing Dr. Levine's remarkable dissertation this afternoon that I was right in my judgment last night in approaching him at whatever stage of the evening it was to ask him to deputize for Professor Loubatières. I am sure that this paper was not the only one for which Dr. Levine could have effectively but he certainly did brilliantly on this occasion.

Likewise I hope you will agree that it was not unreasonable for me to expect that he might have slides on him. In fact I should not have been surprised if he had had slides with the captions in French! He is prepared for almost anything. If I may follow Professor de Duve's example and use a French expression I will say that he met the invitation to *députize* for Professor Loubatieres with complete *sang froid*.

I would like to mention some experiments with sulphonamides that we have had under way in Cambridge for some years. Miss Jeacock is collaborating with me in this work which is necessarily long term. We have been treating cats that have been made persistently diabetic by a short period of treatment with growth hormone (metahypophyseal diabetic cats) with anti-diabetic sulphonamides. I should say that these cats will show in all instances in which they have been followed long enough spontaneous remission from this diabetic condition sometimes after a period of a year or more of untreated diabetes. The glycosuria may be heavy indeed and yet ultimately the glycosuria disappears and the blood sugar becomes normal.

In the few experiments we have been able to do on animals of this sort the administration of anti-diabetic sulphonamides has caused the diabetic condition to clear up much more rapidly than it would have done spontaneously and the problem arises as to what is the mechanism involved. Are these animals diabetic because there is a deficient secretion of insulin by the pancreas? If that is true what has the sulphonamide done? Has it unleashed some inhibiting point in the production of insulin in the pancreas of these animals? We have no very clear ideas on this point. I do not know whether Dr Levine has any ideas in this connection.

I would like to make a comment too while I am here. Mr Chairman about some remarks that Mr Stewart made earlier concerning experiments of my own on the effect of small doses of insulin on ketonuria. I referred to these experiments at a meeting some time ago they were done more than 20 years ago and were concerned with depancreatized dogs which were being investigated as control animals for metahypophyseal diabetic dogs. I was impressed that the metahypophyseal diabetic dogs showed a very substantial glycosuria but no ketonuria. I was able to show that the administration of a very small dose of insulin to depancreatized dogs could completely abolish the ketonuria without any significant effect on the glycosuria or D/N ratio. I could not say that there was no temporary fall of blood sugar level in these animals given the small doses of insulin that we were using but certainly there was no discernible effect on the total magnitude of the glycosuria over 24 hours nor on the D/N ratio. I am not quite sure just what is the significance of those experiments but I think the experimental findings were valid.

STOWERS I have two questions to put to Dr Reid. The first is this

Does he need serum salicylate levels as high as 33-45 mg/100 ml to achieve satisfactory hypoglycaemia? Our own work on a relatively small scale indicates that serum salicylate levels of 10-20 mg/100 ml may be associated with good hypoglycaemia.

The second question is about the B.M.R. effect of salicylates. Does this persist or is it rather transient associated with the loss of intracellular energy stores and the potassium already mentioned? Preliminary results which we have obtained suggest that the B.M.R. effect is transient and not necessarily correlated with the hypoglycaemic effect.

READ: In answer to Dr Stower's first question it is not necessary to maintain high salicylate levels to obtain an effect in blood sugar but we think it is necessary to get a maximal effect and this is what we are trying to find out though it is too soon yet to say what the ideal level is. In reply to the second question the metabolic stimulation shown by increased oxygen consumption is not transient but is directly related to the serum salicylate level. In other words a reduction in oxygen consumption is in our experience invariably accompanied by a fall in serum salicylate level even though dosage is unchanged. This does not happen in all patients but it emphasizes the need to control therapy by estimation of serum salicylate level particularly in the early stages of treatment. We have no information at present on the relation between the hypoglycaemic effect and increased oxygen consumption induced by salicylate.

LEVINE: Since Professor Young raised the question I feel duty bound in a sense since I am substituting for Professor Loubatières to answer as I think he would. There is one mode of action which he has hunted at even in 1946 and 1947 and lately since 1955. That is the effect that the sulphonamides may have on the quick recovery from mild alloxan diabetes. Something analogous perhaps to the quicker recovery from metahypophyseal diabetes. Perhaps the sulphonamides in a partially damaged pancreas act to stimulate the new formation of  $\beta$  cells the duct epithelium.

I wonder Professor Young if you have some histological sections comparable animals treated and untreated with sulphonamides to see that notion is perhaps correct.

YOUNG: I am afraid we have nothing clear-cut on this point at present.

BESSMAN: I should like to call attention to some work that has been done and repeated by a number of people now initially by Fried then by Horwitz and, recently the very elegant studies of H. H. in the *Journal of Clinical Investigation* of the last 6 months on the measuring the blood pyruvate alone as an index of metabolism of things whatsoever. The blood pyruvate is remarkably closely related to the blood lactate and the blood lactate is affected even by insulin. In fact, you can raise the serum lactate concentration by

3 to 4 milli-equivalents by hyper-ventilation. Therefore results obtained with pyruvate levels in patients particularly those treated with salicylate which is known to cause a hyper-ventilation alkalosis as part of its toxic effect could hardly be interpreted as carbohydrate effects.

REID There is one point about salicylate's action in diabetes that might be amplified. We have shown that the more easily a patient is controlled with insulin the better the response to salicylate. Furthermore there seems to be an increasing response to salicylate as insulin requirement rises. This may have a useful practical application in patients requiring large daily doses of insulin who are troubled by hypoglycaemic attacks. Combined aspirin-insulin therapy by drastically cutting insulin requirement may also reduce the frequency of hypoglycaemic attacks.

BUTTERFIELD Although I am acquainted with the experiments referred to by Dr Bessman about pyruvate metabolism, I would like to point out that none of my patients was on salicylates or hyper-ventilating or getting alkaline infusions. The pyruvate changes I described are regularly reproducible and seem to reflect metabolic changes other than the factors Dr Bessman mentioned.

Turning to the mode of action of the sulphonylureas I believe there has been a good deal of confusion about interpretation of peripheral A/V glucose differences measured before and after treatment with these compounds. I hope we shall be able to publish shortly our findings in some experiments measuring peripheral glucose uptake. The results suggest a simple explanation which seems to me to clear up many of the difficulties (Effects of insulin, tolbutamide and phenethylguanidine on peripheral glucose uptake in man *Diabetes* 1958 7 449).

PART IX

CLINICAL ASPECTS OF DIABETES  
AND PRE-DIABETES

Chairman PROFESSOR F G YOUNG



# SOME CLINICAL ASPECTS OF DIABETES WITH RELATION TO INSULIN AND TOLBUTAMIDE

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## VARIETIES OF DIABETES

I should like to start by reaffirming the clinical separation of diabetes mellitus into three types severe mild and secondary. Severe refers to the usually young growth-onset ketosis-prone individual who needs insulin to live. Mild refers to the usually older maturity-onset often obese person who does not become ketonic when insulin is omitted although he (or usually she) may be relatively insensitive to insulin and require 100 or even more units to produce any obvious effect on her carbohydrate metabolism. The dose of insulin being used is no criterion of the severity of the diabetes. Severe and mild diabetes are sometimes called pancreatic and extra-pancreatic respectively on the assumption that the disease in the first case is due to destruction of pancreatic  $\beta$  cells and the second to anti insulin factors which may or may not be monal. However insulin antagonists have been found in the blood stream of the severe diabetic rather than in the mild case whilst islet-cell damage probably always occurs in the pancreas of the diabetic in whom both Ogilvie<sup>5</sup> and Gepts<sup>1</sup> have found an involutionary reduction in amount of islet tissue. Vallance-Owen<sup>7</sup> has shown the insulin-like activity in the blood stream of the mild diabetic of the same order as in a fasting normal person. Thus he does not mean that the pancreatic activity is normal — far from it since an induced rise in blood sugar in the normal to the level that in the mild diabetic produces a several fold increase in insulin activity. Thus the mild diabetic possesses a pancreas which is unable to respond normally to the stimulus of a raised blood sugar level.

It may be noted that the terms mild and severe refer to the evidence of abnormality of metabolism — both types develop the same crippling or killing vascular complications may present similar inheritance patterns and occur together.



same family and both may give evidence of their latent existence by the same sort of abnormal obstetric history and embryopathy in the pre-diabetic phase. It is also true that a mild diabetic may develop ketosis under the influence of certain forms of stress while occasionally a severe diabetic reverts to the mild form. I have twice

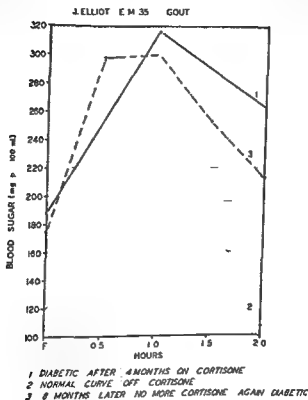


FIG. 1. True diabetes uncovered by cortisone

seen patients in hospital in diabetic coma without any known precipitating factors who were normoglycaemic some months later without insulin. Nevertheless, the clinical difference between these two primary types of diabetes is often neglected. For the use of sulphonylureas with the dosage and

so on are quite useless unless the two diabetic groups have first been clearly separated

Secondary diabetes refers most obviously to that which follows chronic destructive pancreatitis haemochromatosis or total pancreatectomy The diabetes which occurs during pregnancy staphylococcal infections acromegaly or moderate doses of glucocorticoids is not truly secondary but rather the unmasking of an underlying latent or pre-diabetic state The first figure shows a corticoid diabetic becoming apparently normal after cessation of treatment but again diabetic — this time permanently — a few months later

#### PRE DIABETES AND VASCULAR DISEASE

Many of us believe that strictly speaking diabetes begins at birth that the basic abnormality whatever it may be is present for the whole of the lifetime of the diabetic although the overt carbohydrate defect may not be manifest until middle age Time precludes discussion of the arguments in favour of this belief beyond my remarking that the elderly diabetic who attends your clinic may give a history of large babies and stillbirths which extends back 30 40 or 50 years into her pre-diabetic past Furthermore we consider that the specific vascular abnormalities of diabetes are not complications but are an integral part of the syndrome inherited together with the metabolic disturbances Usually they appear later than the hyperglycaemia but sometimes before it They are not a consequence of hyperglycaemia and normalization of the blood sugar even in the very mildest cases is no safeguard against their occurrence It would however appear that they are closely connected with pancreatic damage since true secondary pancreatic diabetes has occasionally led to the later development of specific retinopathy or nephropathy





These various observations are extremely important since they appear to intimate that a defect of pancreatic islet tissue may produce a diabetic vasculopathy which is not dependent upon hyperglycaemia Indeed we have perhaps been over-obsessed with hyperglycaemia as the primary feature of diabetes Insulin has an action in fat metabolism which may possibly be more important than its more obvious effect on carbohydrate It is closely related to the level of the non-esterified fatty acids and stimulates rapid metabolism in fatty tissue It has now been shown to exert an influence

on amino-acid metabolism<sup>3</sup> Might it perhaps have an essential protective effect on blood-vessel walls? A future diabetic might be born with a relatively inefficient  $\beta$ -cell mechanism not so defective that our rough yardsticks of definition based on glucose tolerance can detect it but bad enough so that throughout the years certain blood vessels in certain places become progressively damaged. The special stress of pregnancy may indicate the latent deficiency by producing temporary carbohydrate intolerance in the mother death or excessive size of the foetus and hyperplasia of the foetal islets of Langerhans

#### INSULIN AND PRE-DIABETES

This hyperplasia of the islets of Langerhans in the foetus of a diabetic or a pre-diabetic mother is a remarkable phenomenon. Fig. 2 shows the relative proportions of islet tissue to whole pancreas

#### MEAN ISLET AREAS OF STILLBIRTHS

CONTROLS	 13%
DIABETIC MOTHERS	 65%
PRE DIABETIC MOTHERS	 75%
ERYTHROBLASTOTIC BABIES	 71%

**NOTE** SQUARES INDICATE RELATIVE SIZES ONLY

Fig. 2 Mean proportion of islet tissue in pancreases in different groups

which Woolf and I<sup>9</sup> found on examination of 108 pancreases from stillborn infants. Fig. 3 illustrates a particular pancreas from a foetus of a pre-diabetic mother compared to a normal at the same magnification. From the small amount of suitable material available to us it seems that not only are the islets large and increased in number but they contain an unusually high proportion of  $\beta$  cells and these  $\beta$  cells contain an unusually great concentration of granules. If granules really represent insulin then it would appear that the pancreas of such a foetus may contain up to thirty times as much insulin as normal. What could be the cause of this  $\beta$ -cell hyperplasia? It is not hyperglycaemia because it occurs in the absence of hyperglycaemia in the pre-diabetic. Growth hormone and gluco-



Fig. 11. (1) Plot of otolith in (1) ch

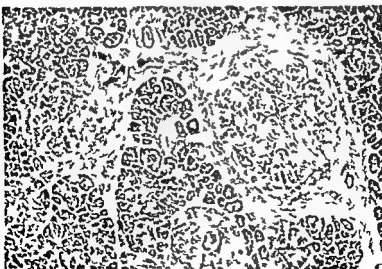


Fig. 12. (1) Plot of otolith in (1) ch



corticoids have been much discussed in connection with embryopathy, but there are cogent arguments against these being the sole or primary villain of the piece. Maternal lack of pancreatic reserve during pregnancy through some mechanism other than hyperglycaemia compensatory hypertrophy of the infant's islets? Now the insulin produced by the foetus might itself act as a growth factor in its own intra-uterine development. Could this possibly be for the excessive size of the diabetic's baby?

If this story has any truth then insulin might be able to prevent the diabetic vascular abnormalities, the foetal and neonatal abnormalities and possibly the actual development of overt diabetes in a pre-diabetic. Certainly and unfortunately insulin will not prevent all vascular disease in diabetics, but there is much evidence when it is given in such a way that good control of glucose metabolism is achieved then the liability to vascular lesions is considerably lessened. Figures from our own clinic indicate the incidence of retinopathy and neuropathy in poorly controlled diabetics was double that in diabetics whose control was good and excellent. Now if our belief regarding the presence of diabetes from birth is true it is surely plain that insulin given only when overt carbohydrate defects are present would not be expected to prevent a lesion which has already been developing for a long time. If we could give insulin early in the pre-diabetic phase might we not prevent the vascular disease? This of course is not known but is worth considering. Methods used in diagnosing pre-diabetes I will not consider here but it may be mentioned that certain family histories make a diagnosis of pre- or latent diabetes mandatory, for instance when both parents are diabetic, when an identical twin is diabetic or in such a family as is shown in Fig 4. Professor Hoet<sup>7</sup> has indeed some evidence that insulin given to a pre-diabetic woman during pregnancy will prevent the development of severe congenital abnormalities, while Wilkerson<sup>8</sup> from Boston has shown that the same treatment will tend to reduce the birth weight of the foetus. Further Hoet has pointed out that the child of a known diabetic mother being treated with insulin seldom becomes diabetic under the age of 10, whereas almost all mothers of children who become diabetic under the age of 6 are untreated latent or pre-diabetics. Insulin given during pregnancy may thus have a protective action on the child. Is it just possible that insulin given to

children in diabetic families would prevent diabetes entirely? It protects the pancreas of animals against alloxan

#### PROPHYLAXIS WITH INSULIN OR ORAL SULPHONYLUREAS

It is admittedly impracticable to suggest that babies or even adults should be stuck with a needle every day without a great deal more evidence of its value. Could the oral sulphonylureas take the place of insulin in prophylaxis? It is very important that we know the mode of action of these substances before embarking on such a programme. If their major action is a stimulation of  $\beta$  cells with th

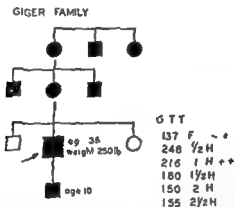


FIG 4 The blacked in figures represent diabetics. The proband (arrowed) had a diabetic son, mother and grandmother. There was no diabetes on his wife & etc. He did not know he was diabetic but the family history led to his having a tolerance test: results as given.

production of insulin then there are two ways in which their use in prophylaxis would differ from that of insulin. First it might be presumed to overtax the already inefficient  $\beta$  cells rather than to rest them. The whole idea of continued stimulation producing permanent structural damage to the  $\beta$  cells is however rather nebulous, has been too readily accepted and finds no proven counterpart in the rest of the endocrine system nor indeed in other organs of the body. Gepts<sup>2</sup> furthermore has found that the damage to islet cells in elderly patients treated with carbutamide or tolbutamide is no greater than that seen in a group of similar untreated or insulin-treated diabetics while in some he found actual regeneration of apparently active  $\beta$  cells. The second difference

between tolbutamide and *exogenous* insulin is that the *endogenous* insulin which is formed under the influence of tolbutamide would be liberated into the portal system and so might really act more physiologically than insulin injected peripherally. Thus Madison and Unger<sup>6</sup> have shown that insulin administered intraportally in dogs produces a much smaller arterio-venous glucose difference than when systemically introduced suggesting a lesser effect on muscle and probably a greater hepatic effect. Tolbutamide does not appear to be toxic in other ways — in fact it seems to be about the most non-toxic drug ever produced. Theoretically it might appear inadvisable to give it to a pre-diabetic or diabetic during pregnancy since we have evidence that the  $\beta$  cells of the foetus are already being excessively stimulated. Although tolbutamide might be called simply a drug of convenience in the treatment of the established mild diabetic it may yet be of true value if used early enough especially in the pre-diabetic.

The advent of the anti-diabetic sulphonamides has taught us more about diabetes as seen in the clinic. In the trial of carbutamide and tolbutamide which we carried out we made it a rule to withhold all insulin from our mild diabetics for several weeks before starting the new drug in order to obtain a proper control baseline. We were surprised to find a number of patients whose carbohydrate metabolism was as well controlled on diet only as it had been with insulin also. In fact in a few the control was actually better without insulin. This has led to a considerable reduction in the number of patients taking insulin in our clinic. Furthermore it clearly indicates that any trial of an oral preparation in diabetes is valueless without periods during which no drug at all is taken. Although insulin was apparently being given unnecessarily to the above-mentioned patients yet perusal of their previous records frequently indicated that it had been prescribed to good purpose when originally administered — 3 or 5 years before. This might be interpreted in two ways either the disease in its natural course had become milder or as I prefer to think the insulin had really had a partially curative effect.

Some clinics in this country have been strongly advocating a return to the use of soluble insulin alone for the control of all severe insulin-dependent diabetics. They claim not only better carbohydrate control but also a greater protection against vascular disease than is afforded by long-acting insulins and in particular the



**lente insulins** This may be so in some clinics but it must be remembered that the long-acting insulins were introduced largely because two injections of soluble a day were inefficient in diabetic control. We have found after some 5 years experience that the lente group of insulins are usually satisfactory and we consider that their wholesale abandonment would be a retrograde step unless more general evidence ■ forthcoming to warrant such a procedure

#### PRIME IMPORTANCE OF VASCULAR DISEASE

Despite the above arguments I do not wish to over-emphasize the potential protective value of insulin in diabetes. Now that coma is a relatively minor problem the importance of diabetes does not lie in its carbohydrate control but in its vasculopathies — retinopathy, neuropathy (assuming that this is basically vascular), nephropathy, coronary and peripheral vascular disease. The blood sugar of itself is a matter of little importance. Although good control of diabetes as measured by glucose estimations may partially prevent the specific diabetic vasculopathies yet it has little or no effect on the development of coronary heart disease (Liebow *et al* <sup>4</sup>). We have observed this in figures from our own clinic. Normally pre-menopausal women are considerably protected from the clinical manifestations of coronary atheroma by the very fact of their womanhood. In this respect diabetes abolishes the advantage of being born a woman. If however the coronary arteries can really be protected by a low intake of saturated fat then surely the very first place in which this type of diet should be advised is the diabetic clinic. If the carbohydrate intake has to be increased and so-called control rendered more difficult is there any evidence at all that this would matter? It seems to me that many 'diabetic diets' being used today are probably atherogenic. As recently as June 1958 a report of the Council on Food and Nutrition (of the United States) recommended that 40 per cent of calories in a diabetic's diet should come from fat. Admittedly a small face-saving and rather ingenuous rider was added with unhydrogenated vegetable oils substituted to a considerable extent for hard cooking fats. Comment on the utter inadequacy of such advice should be unnecessary. There is certainly less evidence that the specific diabetic vascular lesions might be reduced by a lowered fat intake but this is something that must be investigated.

I think that all this may be summarized by the suggestion that

insulin and even possibly the oral sulphonylureas may have a greater potentially protective action in diabetes than we realize but that drugs alone are not likely to solve the real clinical problem of diabetes – the vascular one. We still do not know how best to treat diabetes and are only just beginning to think about preventing it.

I should like to take this opportunity of thanking Professor J F Brock and Dr J H Sheldon for reading this manuscript.

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## THE MEANING OF PRE-DIABETES IN PREGNANCY

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The appearance of the classical symptoms of diabetes mellitus — glycosuria with excessive thirst fasting hyperglycaemia craving for food weight loss acetonuria — does not indicate the beginning of the disease. At that stage the damage of the pancreatic islets — and even other tissues — is usually irreversible.

Tens of years prior to overt diabetes manifestations due to a relative and transient lack of insulin may appear during pregnancy.

Our study has been focused on the disturbance of the maternal environment years before a pancreatic insufficiency i.e. overt diabetes mellitus results. On the other hand the foetal anatomical structures and their development during intra-uterine life may be modified by the lack of insulin in the mother. The impairments in the functions of the child's tissue may only appear later during extra-uterine life.<sup>1 2 8 10 11 13 15 17 19 4</sup>

For many years clinicians have noted the high incidence in the obstetrical history of women over 40 years of age developing manifest diabetes of neonatal mortality stillbirths heavy babies and congenital malformations. Basic research in this field shows that in female rabbits treated with sub-diabetogenic doses of alloxan pregnancy induced a loss of tolerance to dextrose. Ricci<sup>23</sup> describes the increase in islet tissue and lipid infiltration during the last days of pregnancy in rats and also the very notable increase of capillary circulation of the islets during pregnancy. *Post partum* this increased circulation returns to normal. Many islets undergo lipid degeneration and disappear. The number and volume of islets return to normal proportions in 2 or 3 days.

Pregnancy in the woman is a test for islet function. The transient insufficiency which may result and as a rule remains hidden or beneath a cloak is conditioned by the activity of the other ductless glands and in particular by the anterior lobe of

the pituitary the adrenal cortex and medulla the thyroid and the gonads. The placenta as a source of progesterone and ACTH has also to be taken into account.<sup>16, 17, 18</sup>

These premises led to a more systematic study of carbohydrate metabolism during gestation. The relationship between diabetic embryopathy and the changes in the maternal environment of the foetus become evident when the pregnant woman is more carefully studied regarding her glycoregulation and metabolic disturbances.

Wilkerson and Romein<sup>19</sup> in research work undertaken in collaboration with the Children's Bureau United States Department of Health Education and Welfare make a comparative study of the outcome of mothers with and without symptomatic or of pre-diabetes in pregnancy. These symptoms include a obstetrical history, large baby, hyperglycaemia and diabetes in family. This survey allows the conclusion to be drawn that correction of the abnormal carbohydrate metabolism in diabetic mothers can bring about a reduction in the weight of the infants.

A study by Pyke<sup>20</sup> completing the observations of Murphy Munro indicates clearly the diabetogenic role of repeated pregnancies. Also a statistical survey by Beckers and Tuyns of the number of births in western Flanders (Belgium) in 1955 the decisive role of the number of pregnancies and of the age of mother in the development of large infants.

#### I. EXAMINATION OF MOTHERS OF CHILDREN WITH CARDIAC ABNORMALITIES

Downing and Goldberg<sup>21</sup> state that amongst 100 children with ventricular septum defect 31 per cent had a family history of diabetes. Amongst 100 children with inter-auricular septal defect the authors noticed 20 per cent of diabetic heredity by history. There was only one mother who also had an inter-auricular septal defect. Hence the cardiac malformation is seemingly not genetic in origin.

After observing several cases of non-lethal congenital diabetes in children of diabetic or pre-diabetic mothers Hoet<sup>22</sup> and Doyen clinically examined the mothers of malformed children. Twenty-six of the forty-eight mothers examined had features of an endocrinopathy especially diabetes hypothyroidism, or both.

The fact that it is possible to show that an endocrine

present in the mother of a malformed infant, months or the pregnancy leads to the suspicion that an endocrine in pregnancy might play a decisive role in the genesis of formation.<sup>21</sup>

## II. EXAMINATION OF PREGNANT WOMEN FOR CONCEALED DISTURBANCES

In order to observe the earliest phases of endocrinopathy, particularly pre-diabetes we examined those women who had glycosuria in pregnancy or because of a pathology in pregnancy that gave reason for suspecting of pre-diabetes. Our observations concerned 500 mothers, histories were relevant for the birth of heavy infants, of m or still-born children, or for spontaneous miscarriage clinically recognizable cause.<sup>4, 9, 16</sup>

An extensive familial and personal case history was taken complete physical examination performed with special emphasis signs of endocrinopathy and of hypo- or avitaminosis

*In all cases a Glucose Tolerance Test (GTT) was made as follows*

1 In fasting state 100 g dextrose in about 200 c.c water  
 2 Capillary blood was taken and urinary collections made 0 hour 1 hour 1½ hours 2½ hours and 3 hours after dextrose administration

3 Blood glucose was determined by the Hagedorn and method. The presence of true glucose in the urine was looked for with glucose oxidase paper (Clinistix or Testape)

4 The GTT was normal if Blood glucose

at 0 hr 1 hr 1½ hr 2½ hr 3 hr

<1 g <1.80 g <1.30 g <1.00 g <1.00 g %

5 Repeated GTT were performed at short intervals in every woman with a history of perinatal mortality

A basal metabolic rate an examination of night vision by a Goldman-Weckers adaptometer showing haemeralopia and blood examinations for protein bound iodine liver function tests cholesterol and electrophoretic pattern of plasma proteins were performed. A clinical study of 500 women showed their perinatal pathology (Table I)

A summary of the findings in relation to the glucose tolerance



This survey shows that the perinatal pathology of selected cases is frequently associated with a disturbed carbohydrate metabolism in the pregnant woman. The changes in the blood-sugar curve may be minor and have been classified under the heading delayed curves meaning that after 3 hours the fasting blood-sugar value ( $1 \text{ g } \%$ ) was not reached. The blood-sugar values between 2 and 3 hours may be of importance in the evaluation of the disturbed carbohydrate metabolism of the patient. In our selected cases this type of blood-sugar curve was found in 39 per cent of the cases during pregnancy.

This type of data is important as Wilkerson<sup>27</sup> showed in his Oxford survey that 14 per cent of random people with delayed curves became overt diabetics in the same period. This means that one has to be careful in interpreting the findings of blood-sugar curves of pregnant women.

The pattern of the glucose curve during pregnancy is very variable. A normal blood-sugar curve at the beginning of the pregnancy will not necessarily remain normal throughout the pregnancy. A pregnant woman with a delayed curve at the beginning of pregnancy may have a pre-diabetic or a diabetic curve at the end. None of the pregnant women included in this study had a fasting blood sugar above  $1.10 \text{ g } \%$ . Occasionally a pre-diabetic curve of an insulin treated woman will become normal or even flat during or after the pregnancy.

It has to be stressed also that the glucose tolerance test has to be analysed with respect to heredity, the perinatal pathology and the time of the observation in regard to the pathological events. The metabolic disturbances will be concealed especially in cases of congenital malformation or stillbirth as a major endocrine disturbance will not allow a person to become pregnant or to remain pregnant. The characteristic of the pathological picture is that the mother does not complain of any specific symptom, her biological disturbance however is severe enough to affect the developing foetus.

Three clinical histories will illustrate (1) the changing pattern of the glucose tolerance test depending upon the time of observation (2) the beneficial effects of adequate insulin therapy (3) the association of pre-diabetes with other endocrine disturbances which may be as important as pre-diabetes itself.



*Case 1 V L (294)* 27 years old — consulted physician for the first time 2 months after her third pregnancy

In 1952 she delivered a stillborn baby with spina bifida premature at 8 months weighing 3 kg 250 g. In 1953 her second pregnancy terminated in a stillbirth. The foetus weighed 3 kg 250 g — it had a spina bifida. In 1954 a third infant was born alive affected with a meningo-myelocoele hydrocephalus and clubfoot weighing 4 kg 200 g dying at 6½ months

*Family Case History* Maternal grandmother diabetic (six stillbirths)

*Personal Case History* The patient weighed 4 kg 200 g at birth. She had complained of polydipsia polyuria and of sensitivity to cold during her pregnancies. She had frequent conjunctivitis and blepharitis. In 1954 2 months after the last of her three pathological pregnancies her G T T was as follows with 50 g of dextrose

1 03	1 52	1 61	1 36	0 93	
(—)	(—)	(—)	(—)	(—)	(without glycosuria)

Her physical examination showed marked keratoxanthosis of the sole of the feet. She had webbed toes on the right foot

In 1955 the patient was again pregnant and complained about polydipsia and pruritus vulvae her G T T at 6 weeks pregnancy was

0 91	1 78	1 52	
(+)	(+)	(+)	(glycosuria)

She was treated with 10 to 18 units of N P H insulin and 50 000 units of vitamin A daily. Shortly before confinement delivery was induced. The patient delivered a healthy boy of 3 kg 550 g. Ten days after her delivery her G T T was

0 84	1 91	1 93	1 84	1 37	
(—)	(+)	(+)	(+)	(+)	(glycosuria)

One month later her G T T was

0 81	1 59	1 31	1 05	0 90	
(—)	(—)	(+)	(tr)	(—)	(glycosuria)

In 1957 the patient was again pregnant. At 4 weeks pregnancy she had a G T T as follows

0 88	2 08	1 71	
(—)	(+)	(+)	(glycosuria)

Her dark adaptation was 1.2 lower than normal (1.6). She received 20 units of N P H insulin at first and this was increased up to 36 units N P H in the morning and 16 units crystalline zinc insulin in the evening. She was delivered again of a healthy daughter weighing 3 kg 440 g

*Case 2 G R* 30 years old — consulted physician during her third pregnancy because

In 1951 she was delivered of her first baby with congenital cardiac malformation. The boy weighed 3 kg 900 g. In 1953 she was delivered

a second time of a girl with cardiac and renal malformation, weighing 3 kg 600 g. It died 2 days later.

The patient's birth weight was 4 kg. When presenting in 1954 she was in the second month of her third pregnancy. Her G T T was as follows:

0.95	1.91	1.48	1.33
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She was treated with 20 units globin zinc insulin. At 3 months the G T T was

1.31	1.95	1.59	1.34
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She was delivered spontaneously 2 weeks prematurely of a healthy girl of 3 kg 400 g. Six weeks after the delivery the G T T was

0.82	1.78	1.47	1.21
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In 1956 she was again pregnant and was treated again with 20 to 24 units globin zinc insulin. At 6 months her blood sugar curve was as follows:

0.94	1.69	1.64
------	------	------

She was delivered spontaneously of a healthy boy weighing 3 kg 700 g. Six months after the delivery the G T T showed

0.82	1.79	1.50	1.19
------	------	------	------

She was feeling well and did not receive any insulin then.

**Case 3 S M** 29 years old — was first seen 5 days after the delivery of an 8½ months premature stillbirth.

**Past Case History** is relevant for a thyroidectomy for goitre in 1950. The type of goitre remains in doubt.

**Perinatal Case History** In 1951 she was delivered of a healthy girl weighing 3.1 kg. During pregnancy she complained of oedema and occasional vaginal bleeding. Hypertension and albuminuria had been found. In 1958 she was delivered after 8½ months of a stillborn baby. Five days later a G T T was as follows:

0.66	1.72	1.77	1.81	0.63
(—)	(—)	(—)	(—)	(—) (without glycosuria)

Four weeks later

0.80	1.60	0.93	0.87	0.76
(—)	(—)	(—)	(—)	(—) (without glycosuria)

II Met —8 per cent

She was being treated with 100 mg thyroid extract. This case history is relevant for a thyroidectomy and a history of stillbirth. Five days *post partum* the blood-sugar curve was definitely delayed. Four weeks *post partum* the glucose tolerance curve became normal. The patient had hypothyroidism with an associated pre-diabetic tendency during pregnancy.

The close observation of many cases of pregnant hypothyroid women brought the opportunity to follow the progressive deterioration of the G T T as a result of thyroid therapy during pregnancy.

Therefore we consider that when thyroid extract is prescribed and the G T T becomes pre-diabetic insulin has to be given to avoid more profound carbohydrate disturbances. The outcome of five cases of associated therapy with thyroid and insulin are reported (Table VI)

This association is seen even more frequently in recent cases

These three examples illustrate the fact that pregnancy induces quite frequently a diminished tolerance to glucose. The pregnant woman has no specific complaints or does not show any symptom. This disturbance in carbohydrate metabolism may still affect quite adversely the foetal development.

This has been investigated in fifty patients who were previously delivered of children with congenital malformation. Sixteen of the patients were seen in the *post-partum* period of the birth of the affected child. The glucose tolerance tests of these sixteen women are reported in detail.

The glucose tolerance curves in the *post-partum* period may not show evident pathological changes and will not preclude abnormal carbohydrate metabolism during pregnancy. Therefore the glucose tolerance tests in thirty-four mothers of malformed children were analysed during their next pregnancy. The detailed data are tabulated in Appendices A B C D.

Among thirty-four mothers ten had a normal G T T, seven a flat G T T, eight a delayed G T T, and nine a pre-diabetic G T T. The patients with a flat glucose tolerance test had strikingly a rather low basal metabolism for the period of pregnancy.

TABLE III

GLUCOSE TOLERANCE TEST IN FIFTY MOTHERS OF CHILDREN WITH CONGENITAL MALFORMATION

	Total	Glucose tolerance test							
		Normal		Flat		Delayed		Pre-diabetic	
		Total	per cent	Total	per cent	Total	per cent	Total	per cent
Post partum	16	6	38	3	19	5	31	2	12
Pregnant	34	10	29	7	21	8	24	9	26

The data indicate that the birth of a child with a malformation may be associated with a disturbed glucose tolerance in the mother during pregnancy. This disturbance might be transitory and might only be present during pregnancy.

The data show that only 12 per cent of these mothers had a pre-diabetic curve during their *post-partum* period whereas 26 per cent had a pre-diabetic curve during pregnancy (Table III and Appendix E)

A similar observation has been made in women having case histories of stillbirth (Table IV). The pathological disturbances of carbohydrate metabolism are striking among thirty two women eighteen had an abnormal glucose tolerance curve immediately after the stillbirth. During a new pregnancy about the same incidence of disturbances were found (Table IV).

Like the woman delivering a child with a congenital malformation a woman with a case history of stillbirth or miscarriage is very likely to have an abnormal glucose tolerance curve during a next pregnancy — about 40 to 50 per cent of the women with perinatal wastage may show among other endocrinopathies a disturbed glucose tolerance curve when pregnant.

The following data indicate that the correction of the endocrinopathy has a beneficial effect on the outcome of pregnancy. The

TABLE IV  
GLUCOSE TOLERANCE TEST IN EIGHTY-FIVE WOMEN WITH A HISTORY OF STILLBIRTH

	Total	Normal	Flat	Delayed	Pre-diabetic
During pregnancy					
A. Preceding pregnancy ended in stillbirth	27	9.33	8.30	8.30	2.7
B. One of the previous pregnancies ended in stillbirth	26	10.35	2.8	11.42	3.12
During post-partum (after stillbirth)	32	3.16	9.28	9.28	9.28

TABLE V  
GLUCOSE TOLERANCE CURVES IN EIGHTY-THREE PREGNANT WOMEN WITH A HISTORY OF MISCARRIAGE

	Total	Normal	Flat	Delayed	Pre-diabetic
Miscarriage/gravid	22	4.18	6.27	10.45	2.10
Miscarriage/multigravid	22	6.27	3.14	10.45	3.14
Repeated miscarriages	39	7.18	6.15	17.44	9.23
Total	83	17.60	15.18	37.45	14.1

aetiological treatment of the metabolic disturbance influences favourably the foetal development

The observations concern the outcome of pregnancies when mothers with perinatal wastage have been treated with the maximum amount of insulin with thyroid extract (50-200 mg/day) if indicated and with vitamin A (20 000-50 000 units/day)

Pregnant women having delivered previously a child with malformation 34

Pregnancies of the treated women before treatment 69

Pathological pregnancies of the treated women before treatment 35

Successful pregnancies after aetiological treatment 18

Pathological pregnancies after aetiological treatment 0

Hence the untreated pre-diabetic mother as well as the poorly treated diabetic mother is liable to have children with congenital malformation. The aetiological therapy diminishes greatly the risk of such an outcome.

The beneficial effects of endocrine-substitution therapy on the prevention of perinatal wastage are also quite obvious from the results obtained by the aetiological treatment in cases of concealed endocrinopathy with other manifestations of perinatal wastage (e.g. stillbirths, miscarriages).

Table VI summarizes these results on the total number of pregnancies after the aetiological treatment of the metabolic disturbance of the mother.

TABLE VI

RESULTS OF THE TREATMENT OF CONCEALED ENDOCRINOPATHY ON PERINATAL WASTAGE

	Pre-diabetic pregnancies		Hypothyroid pregnancies		Pre-diabetic hypothyroid pregnancies	
	Before insulin R	With insulin R	Before thyroid R	With thyroid R	Before thyroid + insulin R	With thyroid + insulin R
Number of pregnancies	95	35	38	14	32	5
Per cent of normal pregnancies	0 /	77 /	19	70	13	■
Per cent of pathological pregnancies	48	3	72 /	7 /	74	20 / *
Per cent of foetal macerations	32 /	20	9	3	13 /	0

\* The single pregnancy terminated in a stillbirth—Rh incompatibility was associated with concealed endocrinopathy. A high titre of anti-Rh bodies was present in the mother's serum.

## III THE EFFECT OF INSULIN ON DEFECTIVE DARK ADAPTATION AND CAROTINODERMIA

*Relationship between Disturbances of Carotene Metabolism during Pregnancy and Foetal Pathology*

(In collaboration with Dr F Stein)

A factor common to the pre-diabetic and hypothyroid pregnant woman is carotinodermia and defective dark adaptation. The latter is measured by the adaptometer of Goldman-Weckers. In a pre-diabetic patient the defective dark adaptation will improve by insulin treatment. The following cases illustrate this statement.

**Case 1 L S (Fig 1a)** A 29-year-old married male father of one consulted his physician because of increasing fatigue and somnolence over the last few months. His father was a known diabetic for the last 5 years of his life and he had been treated with insulin.

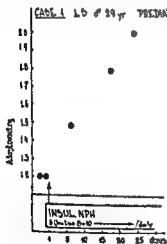


FIG 1a

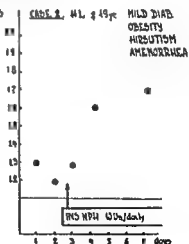


FIG 1b

Patient's physician found glycosuria and referred the patient to the diabetic clinic for a glucose tolerance curve (100 g dextrose with a blood sugar every 45 minutes) 0.83 - 1.33 - 1.9 - 1.1 - 1.07 without sugar or acetone in the urine. The glucose tolerance test was not pathological.

notwithstanding the blood-sugar did not reach fasting levels within 3 hours after glucose intake

The adaptometry\* (measurement of night blindness) was low 1.2 at two occasions after 7 days of treatment with 6 units of insulin NPH the adaptometry rose to 1.5 and 10 days later with 8 units of insulin NPH to a normal value of 1.8 Twenty-three days after insulin treatment the adaptometry was 2.0

*Case 2 H L (Fig 1b)* A 19-year-old girl was referred to hospital because of obesity hirsutism and amenorrhoea She weighed 87.6 kg height 1.57 m No major metabolic abnormality could be found but a pathological glucose tolerance curve (100 g glucose with a blood sugar every 45 minutes) 1.03 g/l - 2.49 - 1.13 - 1.65 - 1.30 with sugar in the urine

The adaptometry was 1.3 repeat 1.2 before insulin treatment 24 hours after insulin treatment adaptometry was still 1.3 3 days after treatment it was 1.6 and 5 days after 10 units NPH daily it was 1.7

*Case 3 A V (Fig 2)* A 32-year-old mother of two is referred to the hospital because of myxedema She had been treated for diagnosed hyperthyroidism with radioactive iodine 6 years ago

At the time of entry her subjective symptoms and physical examination were classical for myxedema She was characteristic for marked xanthoderma and paleness Her adaptometry before treatment was 0.9 She was then treated with tri-iodothyronine in increasing dosage 25  $\mu$ g 50  $\mu$ g 75  $\mu$ g a day Her adaptometry rose quickly up to 1.2 After 1 month on 75  $\mu$ g tri-iodothyronine a day the adaptometry was 1.6 The patient was then started on thyroid 50 mg a day Her adaptometry was still 1.6 after 2 weeks of thyroid

We consider that this improvement of the vitamin-A genesis at the level of an effector organ the retina was an indication of some of the events at the level of the foeto-placental tissues

Hale and Warkany Wilson and Schappenberg described hypovitaminosis A as a teratogenic condition We consider that pre-diabetes and hypothyroidism produce an insufficiency of vitamin A at the level of the effector organ with the greatest need<sup>4 9 14 6</sup>

Recently Millen and Woollan<sup>18</sup> have seen that experimentally hypervitaminosis A has a teratogenic action in the rat This teratogenic effect was definitely potentiated when cortisone was injected during

The measurement of dark adaptation (adaptometry) is expressed as the logarithm of the light intensity in lux, that one is able to detect in 25' after a prolonged exposure to bright light Normal values are above 1.6 The values under 1.6 are abnormal and they show a poor capacity of dark adaptation

pregnancy In complementary experiments they demonstrated a close insulin-cortisone relationship in experimental teratogenesis The experiments showed that insulin prevents wholly the potentiating action of cortisone on the teratogenic effects of hypervitaminosis A

Experimental hypothyroidism during pregnancy as the result of administration of 4 methyl 2 thiouracil also has a potentiating effect on the teratogenic activity of hypervitaminosis A

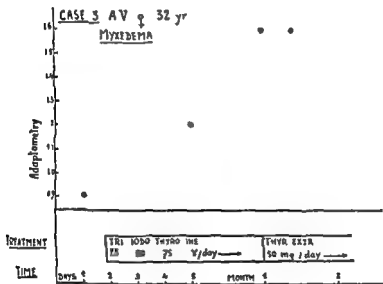


FIG. 2

Therefore we underline the normalization of the dark adaptation as the result of insulin treatment The improvement of the nutrition of the placental and foetal tissues was shown by the outcome of the pregnancies in those women with a long record of pregnancy wastage

These facts point to an action of insulin and thyroid extract on a metabolic pattern involving carotene and its dynamic equilibrium with biologically active vitamin A The gross and permanent disturbance of glucose tolerance test is a later symptom of the progressing disease



The link between pre-diabetes in pregnancy and the foetal pathology and the congenital malformations is not a direct one. Some more specific damaging factors other than hyperglycaemia may cause the embryopathy. The example of the disturbance of the vitamin-A metabolism is probably not the only one.<sup>14</sup>

The maternal environment of a concealed pre-diabetic mother may be unfavourable for the foetus through more than one factor. The moment and degree of the pathological metabolic disturbance will determine the nature of the events. Early in pregnancy it may be a miscarriage, later a stillbirth with gross anomalies. In milder cases a liveborn baby may just show an adaptation crisis to extra-uterine life. In other cases a live newborn may have a pancreas with numerous hyperplastic islets (Dubreuil *et al*<sup>35</sup>). The overweight newborn with a Cushing aspect is an easy retrospective fact in the clinical history.<sup>8</sup> The anatomical lesion of the foetal pancreas is a sign of embryopathy rather than the exclusive result of genopathy.

Careful studies may produce an explanation of the phenomenon of anticipation of diabetes.<sup>5</sup> The natural history of infantile and juvenile diabetes must be considered in the light of possible intra-uterine pancreatic changes. A foetus carrying a diabetic gene may be further damaged by a disturbed maternal environment. For instance, the offspring of the diabetic father may be submitted to an unfavourable maternal environment of a concealed pre-diabetic mother.

On the other hand, non-lethal congenital malformations in the offspring of diabetic parents or the association of all varieties of anomalies with clinically more or less severe diabetes mellitus points to embryopathy. We focus the attention on observations of syndactylus, situs inversus, renal malformation, haemangiomas, bifid uterus, mongolism, Klippel Feil syndrome and supernumerary nipples associated with diabetes mellitus.

### CONCLUSIONS

From a practical point of view, the glycosuria and the G.T.T. during pregnancy may not be isolated from the other clinical data. To detect concealed metabolic disturbances which may have considerable effects on the foetus, it is of importance

- 1 To acquire a maximum of gravido-puerperal data of the

pregnant mother's relatives in particular birth weight stillbirth or congenital anomalies of sibs

2 To look for minor clinical signs expressing metabolic disturbances like xanthosis keratoderma cheilosis glossitis anaemia acetonuria night blindness emesis pruritus vulvae excessive weight gain or loss

3 To establish the association of pre-diabetes with other endocrine pathology hypothyroidism is frequently associated with pre-diabetes (importance of goitre) Virilism may point to adrenal disturbances

4 To repeat G T T if perinatal pathology has been severe (stillbirth) Protein bound iodine electrophoresis and basal metabolism will be checked regularly

5 To be alert about endocrinopathies in the parents which may point to possible foetal pathology

#### SUMMARY

Pre-diabetes during pregnancy has been discovered in a clinical survey of mothers of malformed children or with pregnancy wastage syndromes

Covering 500 cases of women selected on these grounds it may be stated that 50 per cent present disturbances of the G T T

These symptoms show up by predilection during pregnancy. Minor alterations before pregnancy become frankly pathologic during gestation

Hypothyroidism may be associated with a disturbance of carbohydrate metabolism.

These studies gave the opportunity to treat eighteen pregnant women affected with endocrinopathies

The pregnancy wastage for the previous gestations reached thirty-five out of sixty nine

Eighteen pregnancies carefully treated and controlled gave live newborn without a single loss

Attention is focused on the intimate relationship of pre-diabetes or hypothyroidism with clinical signs of hypo-vitaminosis A. This might be one of the metabolic factors of the teratogenic action of the minor endocrinopathies of pregnant mothers. Incomplete treatment of overt diabetic women results in the same foetal pathology

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This work has been performed with the technical assistance of Messrs L Rutten G Crombez and Miss H Hottelet

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### CONCEPTUAL INFORMATION

N	Anemone		Placental pregnancy										
	Nl format cm	Bark weight kg	C <sub>20</sub> da	Cann <sub>d</sub>	Wash for %	Glucose & lactose %				BMR	V <sub>1</sub> 4 y	Adipo Nit 16	
						g	g	g	g				
100	Polymeric kidney	4 000	1st	3rd	8th	0.87	1.42	1.07	1.18	0.61	+27		
81	Longitud. 1 x h p	2 200	1st	2nd	nd	0.76	1.19	1.00	0.91	0.89		18	
103	Little d waste	8 m	1st	3rd	4th	0.75	1.30	1.27	1.04	0.66			
03	Card. malform.	3 000	1st	3rd	7th	0.82	1.34	1.1	1.09	1.21			
23	Card. malform	3 300	1st	3rd	nd								
	Spiral buds												
	Meso gonocite	2 850	1st	2nd	3 d	0.67	1.45	1.20	1.15	0.73	+26	37	08
410		3 000	9th	11th	6th	0.73	1.35	1.18	1.4	0.82	+27	103	19
303	Anomorph. 1	2 7 m	1st	3rd	nd	0.76	1.25	1.10	1.02	0.8			
463	Unknown mal	1 700		2nd	4th	0.86	2.15	0.70	0.51	0.74	-23		
						1.7							
406	Tu m	2 600	1st	nd	3 d	0.79	1.17	0.9	1.00	1.7			
	Cardiac malform	3 000											
	Deceph. stenou	3 000	nd										
48	Spiral buds	3 000	nd										
	Spiral buds	3 610	3rd										
	Spiral buds	3 610	4th	5th	4th	0.83	1.1	1.19	1.10	1.07	-12		



### Abstract C

CELLS COME TOBACCO THE DURING PREGNANCY OF TWENTY-FIVE WOMEN HAVING PREVIOUSLY DELIVERED A CHILD WITH CONGENITAL MALFORMATION

C Delayed curves: 1

[illegible]



# APPENDIX B

GLUCOSE TOLERANCE TEST DURING THE POST-PARTUM PERIOD OF SIXTEEN PATIENTS AFTER THE DELIVERY OF A CHILD WITH CONGENITAL MALFORMATION

N	Ala Conatus	Birth weight kg	Cap- sule	Age months	Glucose tolerance test					Vol. A Y	Ad No No 1.6
					gms. per						
					0	1 hr	2 hr	3 hr	4 hr		
A Normal G T T 6											
132	Hydrocephalus		1st	26	0.86	1.31	1.26	0.93	1.05	37	
184	Spona bil da Spona bil da	3.450	1st	23	0.69	1.26	0.98	0.81	1	18	
410	Cyclops	2.100	2nd	24	0.83	1.49	1.08	0.87	0.76		
418	Anencephalus	3.000	2nd	24	0.90	1.27	1.09	1.01	1.08		
36	Sers undiffer										1.1
149	Cub feet	2.350	3rd	27	0.74	1.50	1.20	1.18	1.03	64	
	Harel p	3.700	1st	24	0.83	1.76	1.15	1.08	0.65		
B Normal G T T 5											
410	Harel p	4.480	2nd	33	0.81	1.04	0.79	0.83	0.56		
460	Def. palus	3.200	2nd	25	0.92	0.99	0.91	0.70	0.67		
505	Meningocele	p	2nd	29	0.86	1.13	0.97	0.93	0.2		0.9
C Delayed G T T 5											
37	Hydrocephalus		1st	25	0.92	0.94	1.70	1.13	1.41		
41	Meningocele	3.250	1st	24	0.63	1.1	1.32	1.30	1.18		
370	Spona bil da	p	1st	25	0.8	1.49	1.1				
452		1.700	1st	36	0.86	1.62					
51	Anencephalus	2.750	4th	24	0.81						
D Pre-diabetic G T T 2											
30	He. lip										



## DISCUSSION

TUNBRIDGE Professor Young in opening the meeting suggested that we should let our hair down and thus I shall proceed to do as far as nature permits! After the excellent presentations by Dr Jackson and Professor Hoet a clinician may be permitted to ask a series of questions (1) What do we understand by the term diabetes mellitus? (2) What do we mean by control? (3) What are the causes of the so-called complications of diabetes mellitus? And lastly (4) How may they be prevented, since they provide the major problem for the clinician treating diabetes mellitus today?

Diabetes mellitus is really an umbrella term used to cover all cases exhibiting hyperglycaemia. A raised blood-sugar level is the lowest common denominator for the making of the diagnosis. Despite numerous attempts to classify diabetes either according to insulin dosage or insulin resistance or sensitivity the age of the patient or according to somatotype no satisfactory clinical classification has yet been devised.

The problem as Dr Jackson so clearly indicated, is whether an abnormal glucose tolerance test indicates pre-diabetes or diabetes mellitus. The majority of clinicians will accept that a patient presenting with a history of sepsis and ketosis even if at a subsequent date they had a normal glucose tolerance test should be considered either as a pre-diabetic or as a potential case of diabetes mellitus. The interpretation of minor differences in the glucose tolerance test is often a matter of opinion and therefore conclusions as to the significance of these changes are very difficult to interpret. The follow-up of cases showing minor degrees of abnormality in a glucose tolerance test over a period of at least 10 years have been very few and such reviews as have been carried out would indicate that rather more than half of such patients ultimately develop diabetes mellitus but by no means all of them. The problem for the clinician, is how to make an accurate prognosis in the absence of any family history of diabetes. The recently introduced cortisone test may assist in the differentiation but it is too early yet to say how reliable this will prove. To revert to the patient who has had an initial incident of glycosuria and after treatment had apparently maintained normal health for 20 years but who then presents with severe arterial degeneration and retinopathy. Has he had active phases of diabetes over the 20 years which have been undiagnosed or has he had a silent hyperglycaemia for 20 years? In other words is hyperglycaemia the only criterion of diabetes mellitus or are there others which at the present time we have no means of assessing and are therefore overlooked? This raises a most important problem and one which is at the root of any policy that we must adopt concerning both the diagnosis and treatment of diabetes mellitus. When we come to consider the problem of the control of diabetes mellitus we

are on difficult ground because we do not know what we are. There are excellent reports of short term control in hospital diabetic wards but there is very little satisfactory work on the trial. In Sweden they carried out a survey by questionnaire in opinion a very satisfactory method. They wrote to 5,07 patients and asked them whether they had followed their diet. Thirty-six per cent indicated that they had followed the diet well, 54 per cent admitted they had not followed it very carefully, 10 per cent not at all. I tried a simple experiment in my own clinic a random sample of those living in the city of Leeds largely because of convenience of visiting. The random sample numbered one hundred five and ninety four of these patients co-operated. In sixteen followed exactly as one should expect in thirty-six the diet was satisfactory but in the remainder hopeless. In Arthus Lundbeck went into the problem and analysed very carefully all the factors concerned. I think we can say that the most important of all is that of direct personal supervision. We should assume that at best only 15 per cent of our patients however well we teach them and how frequently we see them will really adhere to their diet over a long period of time. Another problem in assessing the effect of diet is the fact that fashions change and over the last 40 years there have been considerable changes in the approach to the dietary control of diabetes.

What is often not realized are the wide changes which may take place in the blood-sugar level during the 24 hours even in a patient who appears to be satisfactorily controlled. My colleagues and I have already drawn attention to this in assessing the insulin requirements and it is not at all uncommon for a sensitive diabetic to have short periods of ketosis lasting for a quarter or half an hour associated with a blood sugar of 200-300 mg per cent and yet during the remainder of the 24 hours have a blood-sugar level below 200 mg per cent. It is these facts which make using the blood-sugar level the principal indication of adequate controls so difficult.

This leads to my third point the question of the relationship of the so-called complications of diabetes mellitus to the control of the hyperglycaemia. Dolger in 1947 first hinted that the control of the hyperglycaemia did not make any difference to the development of complications. The Boston school have always stressed the importance of good control and have published many papers suggesting that good control was followed by a lowered incidence of complications. Lundbeck, in his very careful follow-up of children in Arthus went no further than to say that the evidence suggested that retinopathy which is really the only objective complication we can diagnose early was significantly less in those whom they assessed as having been well controlled.

In discussing the whole problem of complications I think it is necessary

to divide them into two groups the retinopathy neuropathy and nephropathy which seem to be specific for diabetes and the widespread vascular degeneration which histologically is not different from that found in old age. The only possible difference is that in diabetes it is found very widespread and in veins as well as arteries. The specific nature of the first group is perhaps illustrated by the following case: the patient was first seen at age 30 when she had had diabetes for only 5 days. She was a school teacher and very co-operative and during the 4 years that I attended her she was extremely careful with her diet and never to my knowledge had a blood sugar over 400 mg per cent until just before her death. Retinal haemorrhages were first observed 3 years after the onset and she died 18 months later having developed severe retinitis proliferans and severe renal lesions. The post mortem examination revealed extensive Kimmelstiel-Wilson changes but only slight evidence of atherosclerosis. This patient seemed to illustrate the specific nature of certain vascular changes found in association with diabetes mellitus and to emphasize the point I have already made that there are other disorders in diabetes mellitus besides hyperglycaemia for which at the present time we have no reliable method of assessment.

On the other hand in the elderly diabetic one does find a very high incidence of coronary disease and arterial degeneration. In other words it seems as though senescent changes were occurring a decade earlier than in normal people. In a series of nearly 400 autopsies in which I compared the gross findings in elderly arteriosclerotics in people who had had glycosuria discovered for the first time just before death and in those who were known to have had diabetes for many years there was no gross difference in the incidence of atheroma in the major vessels of the heart or the brain.

This brings me to my final point namely that of prevention. The acceptance of the views of Dr Jackson and Professor Hoet would be valuable because it is conceivable that if we could control hyperglycaemia we might lessen the present-day clinical picture in the treated diabetic and possibly lessen the high incidence of complications. On the other hand there is considerable clinical evidence that hyperglycaemia is by no means the whole story and we should be on our guard against accepting hyperglycaemia as the only metabolic abnormality in diabetes mellitus.

Before closing I would like to pay a tribute to our hosts. It has been very stimulating to clinicians to hear of the experimental work being done in relation to diabetes mellitus and naturally we may find it difficult to apply immediately the information we have gained. Professor Young has said that we have been possibly a little removed from the treatment of patients but I think that he and all of us may rest assured that we have been dealing with a very important problem because in Great Britain

alone within the next 25 years nearly 1 per cent of the pop likely to develop diabetes mellitus although we shall probably made the diagnosis in about half the cases

OAKLEY I just want to make two points both to emphasize h I agree with Professor Tunbridge about two points he made on the subject of the classification of diabetes into clinical types this has been very useful but that it can be overdone I think it very hard and fast classification which could be regarded as sati and that any grouping of diabetics leaves a great deal of interme who cannot conveniently be fitted into any one particular gr mention this because I think we are apt to forget what I regard as a important observation which was described I think by Dr Jones — sorry I am not quite sure of his name He described a group of ca the West Indies in which he had these young diabetic patients who highly insulin resistant who showed not the slightest tendency to b and were a complete reversal of the accepted picture in Europe Follow that recently I have had a report from Ceylon of a series of cases exhibiting exactly the same manifestations and it is rather significant the two groups have very much the same and rather peculiar and unig feeding habits I think this tendency to force diabetics into certain gories is liable to make one overlook those groups which do not fall i any such category and such groups I think will very greatly reward n work and study

The other point I wanted to make was regarding arterial complications I was delighted to hear Professor Tunbridge say what I myself have found in a follow up of 2460 patients in my clinic in which we studied the incidence of peripheral arterial disease and we found no correlation whatever between the duration of the disease the severity of the disease and the presence or absence of peripheral arterial lesions We did, how ever find a significant and consistent relationship between such disease and the age of the patient I would agree entirely with him that we are probably dealing with two separate entities when we are considering the specific retinal and renal lesion and the lesion in the lower extremity which by no means that I myself know of can you distinguish from semic arterial change

LEVINE I should like to draw your attention to a piece of work which was done by a Japanese pathologist The results have been bothering me ever since I read the paper and I would like especially those people like Dr Jackson and Professor Hoet, who are interested in hereditary and congenital aspects to throw some light on such data

Dr Okomoto was interested in the relationship of alloxan to the zinc content of the islets, and he worked on a theory of zinc complex formation as a mode of alloxan action Having few rabbits to work with, he

used rabbits that had recovered from alloxan diabetes or those that were not successfully alloxanized. He bred them in order to produce more rabbits — a very worthy enterprise. To his astonishment he found that when the progeny grew up they required much less alloxan to become diabetic than did the fathers and mothers. In the F<sub>3</sub> and F<sub>4</sub> generations there was a 30 per cent spontaneous hyperglycaemia and glycosuria. Being a pathologist he measured the volume of the islets and there was a consistent decrease from one generation to the next. Since he came from Japan and not from Lysenko territory I was puzzled about this.

I have been urging people in the United States and elsewhere to let a geneticist have a go at it. Similar observations have been recorded by German observers in 1952 in studies on alloxanized rats. I should like to hear Dr Jackson's and Professor Hoet's comments on these papers.

**SMART** Work which was carried out in the Royal Air Force during the war did not indicate that vitamin-A deficiency was a limiting factor in raising the dark-adapted rod threshold. Groups were observed who were receiving supplements of vitamin A but who nevertheless had raised dark-adapted rod thresholds. Also substances such as alcohol which alter the pupil size can alter the value for the dark-adapted rod threshold as it is usually measured. I have measured the dark-adapted rod threshold in many cases of steatorrhoea in which condition there is malabsorption of vitamin A and I have not found it to be significantly raised though this is possibly because sufficient B-carotene may be absorbed.

Can Professor Hoet tell us the effect of normal pregnancy and of giving insulin to normal people on the dark-adapted rod threshold?

**JACKSON** In agreement with Professor Tunbridge and Dr Oakley certainly we must control our patients' blood sugars at the present time but I tried to suggest that there may be other things more important to control which we do not yet know. I am similarly in agreement that the exact categorization of all diabetics is impossible but nevertheless the general concept of the classification as I have outlined is very important for many reasons.

As regards the validity of the diagnosis of pre-diabetes I would point to our own small series of women who gave rise to infants with enlarged pancreatic islets — seven out of twelve of them are already diabetic after 5 years while of those diagnosed in 1952 on the basis of obstetric suspicion and slightly abnormal glucose tolerance all five of those whom we have been able to follow up have become diabetic within 5 years. There are several other published papers illustrating the progression of pre-diabetes to overt diabetes including the recent work of Fajans and Conn and of Wilkerson. I believe that temporary diabetes is merely a phase in the life history of a diabetic when some particular

to light the latent pre-diabetic abnormality. Patients who state must be considered to be potential permanent diabetics and treated accordingly.

Professor Young asked for a definition of pre-diabetes. While I really think of any real dividing line between pre-diabetes and diabetes, yet for general discussion we keep them distinct. I will try a definition. The state in which evidence of carbohydrate abnormality is not clinically apparent, but in which certain rather specific stresses produce certain specific abnormalities which we know to be related to the later development of overt diabetes.

Finally Dr Levine's extraordinary animals which appeared to be neo-Lamarckism. It is conceivable that the alloxan had damaged the pancreas subclinically so to speak so that the extra stress of pregnancy rendered it partially inadequate. The abnormal intra uterine environment then produced damage to the foetal pancreas (perhaps as in the human) so that one could thus imagine the appearance of diabetes in later generations. This is merely a hypothetical alternative to a genetic one.

Young: Thank you Dr Jackson. I think I was rather intrigued by the statement in your abstract that diabetes really exists from birth — that is pre-diabetes is it?

Jackson: In ordinary terminology yes.

Hoet: Three points may be commented on as a result of this discussion.

1. Professor Tunbridge brought forward in some way an opposition between some form of transient diabetes and permanent diabetes. This is the problem of the beginning of diabetes mellitus and their relationship with the classical clinical picture of irreversible permanent overt diabetes mellitus accompanied by more or less severe complications. From another viewpoint, it is the question when has diabetes mellitus in a transitory form to be controlled with insulin. Is it possible to prevent the irreversible metabolic decompensation? We consider each metabolic disturbance caused by an infection, a mental stress, trauma or burns as due to some transitory insufficiency of the islet function. This is also the case in mild forms of pre-diabetes during pregnancy. Although transitory diabetes may be recovered from without any anomaly of the G.T.T. it appears to us that it has to be considered as a primary phase of diabetes mellitus although it may proceed for more than 20 years before overt irreversible diabetes mellitus results. Therefore dietary and insulin treatment is justified in those pre-diabetes phases more than they appear frequently in the offspring or subs of more severe diabetes. We suggest that immediate control of any mild disturbance of carbohydrate metabolism has to be put in action, so as to shorten the decompensated phase and to prevent further evolution to more severe and longer periods of diabetes mellitus. For each known case of diabetes mellitus there is a

patient with a concealed form waiting for some or other complication to be a recognized case. Our treatment must protect the cases of potential diabetes.

2 Dr Levine did mention the experiments of Japanese investigators on the offspring of alloxanized pregnant rats. The unfavourable maternal environment has produced newborns which later in life become glycosuric even in the offspring of the second generation. Bartelheimer and Kloos (*Zeitschr für die Ges Experim Medizin* 1952 119 46-65 *Die Auswirkung des experimentellen Diabetes auf Gravidität und Nachkommenschaft*) have seen hyperglycaemia and glycosuria in some cases with gigantism in second and third generations of null alloxanized mothers. Dr Haumont in experiments in collaboration with Goormaghtigh and Hoet has treated pregnant rats with rather high doses of cortisone from the eighth to the eighteenth day of gestation. A percentage of resorptions are seen but in the living offspring urogenital malformations in the females were observed. In the following generation this means rats born of mothers not treated but being the daughters of cortisone-treated rats. Haumont has seen spontaneous cataracts and after 6 to 8 weeks hyperglycaemic phases occurred see preliminary paper in *Annales d'endocrinologie* 1959 19 No 2 pp 442-5 *Effets de la Cortisone administrée pendant la gestation*.

Therefore we stress the importance of a normal favourable maternal environment in every case but more so when some pathological genetical trend may be suspected.

3 A colleague of Manchester did recall that night pilot candidates were investigated about their night vision. On nutritional deficiency it has not been easy to discover clear cut cases of night blindness. Environmental factors or pupil dilatation or use of alcoholic beverages may play a role in ascertaining the dark-adaptation level. We have been studying metabolic carotene disturbances depending on insulin deficiency or hypothyroidism. There was no nutritional deficiency. We demonstrated the correction in one and the same patient of the defective night vision by aetiological treatment with insulin or thyroid hormone. The favourable response was seen in 3 days in selected fresh cases of pre-diabetes. This insulin action is seen also in pre-diabetes of pregnancy. It is an indication of a metabolic action of the hormone which is of paramount importance for the placenta and foetal tissues and harmonious foetal development.

## CONCLUDING REMARKS

F G Young

YOUNG The last item on the programme is General conclusions by F G Young I am very hesitant general conclusions from the last two days but I think number of points that have emerged which strike particularly interesting

One must necessarily be humble as the amount that know about insulin and diabetes but I recall Clara remark that what we believe we know is often a hind knowing the truth I and perhaps others have found it understand a good deal of what I thought I knew before it

A point that has interested me very much in the discussion have had is the number of times the mechanism of action has been linked to high energy nitrogenous phosphate This is an idea which is clearly in the minds of several workers and it may well be that it will be a most fruitful years to come But as Dr Cori said we know little or nothing the mechanism of sugar transport and little or nothing about mechanism of action of insulin We must still agree after 2 matters of this sort for two days that this is still true

On the other hand we have agreed about much We have the work of Dr Moloney on the production of insulin antih supported confirmed and used as in other laboratories while it is considerable agreement as to the importance of insulin in biosynthesis It is clear too that the existence of the insulin which are being investigated is of fundamental significance in the growth of knowledge of the mechanism of action of insulin and of diabetes and that these substances complicate the biology of insulin The bio-assay of this hormone is a matter that needs more further investigation before it is on a satisfactory basis But many of different sorts are being brought to bear on this problem and knowledge grows particularly as knowledge of the action of insulin antagonists grows so I think we shall have more understanding of the cause of diabetes and the mechanism of action of insulin

I very much enjoyed the paper on salicylates and aspirin by Dr



Reid It is not unreasonable to say that one of the important results of the discovery by Hench of the remarkable effect of rheumatoid arthritis has been that for the first time a thorough investigation of the therapeutic effects of salicylates has been made. Salicylates had been available for 80 years or more although thorough clinical trials of their actions and effectiveness had not been undertaken until recently and as Dr Reid said the more is known the more we are astonished at the effectiveness of this comparatively simple chemical molecule salicylic acid and its simple derivative aspirin.

I would like to take the opportunity of expressing the appreciation of us all to the two gentlemen who provided papers as substitutes Dr Beigelman and Dr Levine. I am sure that those of us who know Professor Loubatières will very much regret his absence and its cause but his research has been most ably surveyed by Dr Levine.

I would again like to thank our hosts for having made this meeting possible. Dr Hobday mentioned in his opening address that I had been concerned in the proposal that this meeting should be held in this connection. I rather feel like the man who said to his neighbour in a London Club 'I had no difficulty at all when we had twins!' It has been no difficulty at all to me but I would like to emphasize how hard Dr F. W. Wolff in particular has worked on the arrangements for this meeting. He has written innumerable tactful letters and he has handled all the arrangements remarkably effectively. I have discussed matters with him once or twice but he and of course the Committee of the British Insulin Manufacturers behind him, have been responsible for all the details in a most effective manner.

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